ROLE OF NEUROTROPISM IN HIV-1 GP120 INDUCED OXIDATIVE STRESS AND NEURODEGENERATION

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Abstract

An estimated 50% of individuals with long-term HIV infection are affected by the onset of progressive neurological and cognitive complications referred to as HIV-associated neurocognitive disorders (HAND). While the molecular mechanisms underlying pathology in HAND remain poorly understood, synaptodendritic damage has emerged as a hallmark of HIV infection of the CNS. This damage is likely mediated by a combination of indirect mechanisms involving the release of inflammatory mediators and viral proteins from infected glial cells, and direct effects mediated by the interaction of neurotoxic viral proteins with neuronal receptors. The neurotoxic HIV envelope glycoprotein gp120 interacts with neuronal receptors CCR5 and CXCR4 to induce the coalescence of lipid raft domains into large, stable platforms— a proposed mechanism for clustering components of receptor-activated signaling cascades. The interaction of proteins with lipid-raft localized receptors as a mechanism of regulating pathologic signaling has been observed in other neurodegenerative diseases, most notably in Alzheimer's disease, where amyloid-β (Aβ) oligomers interact with lipid raft-anchored cellular prion protein PrP^C to activate a pathway leading to the formation of cofilin-actin rods-like inclusions (rods) in neuronal processes. Rods have been linked to synaptic dysfunction via sequestration of cofilin and the disruption of vesicular transport resulting from the occlusion of neurites in which they form. Given similarities in neuronal response to gp120 and Aβ, we sought to assess the ability of gp120 to induce rods. Here, we report viral envelope protein gp120 induces the formation of cofilin-actin rods in E16 mouse hippocampal neurons via a signaling pathway common to oligomeric, soluble amyloid-β and inflammatory cytokines. Our studies demonstrate gp120 binding to either chemokine co-receptor CCR5 or CXCR4 is capable of inducing rod formation and signaling through this pathway requires the NADPH oxidase-mediated formation of superoxide (O_2^-) and the expression of cellular prion protein (PrPC). These results link gp120-mediated oxidative stress formation to the generation rods in a previously undescribed mechanism of early synaptic dysfunction observed in HAND.

Dedication

In loving memory

Patrick James Smith

1986-2017

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Chapter 1: General Introduction

Human immunodeficiency virus (HIV), a member of the lentivirus genus of the Retroviridae family of RNA viruses, is the etiologic agent responsible for acquired immune deficiency syndrome (AIDS) and its associated complications, which has been responsible for 35 million deaths and more than 70 million infections worldwide since first emerging in the early 1980s (1). As with other lentiviruses, HIV causes chronic disease in infected individuals with a characteristic period of clinical latency and persistent viral replication. Advances in understanding the virus and improvements in diagnosis, treatment, and outcomes of disease have led to a substantial reduction in HIV-related mortality. However, as the epidemiology of HIV/AIDS shifts from an acute infection to chronic disease, new challenges emerge in terms of management of long-term infection and associated comorbidities that can have profound impacts on quality of life for infected individuals. HIV isolates are grouped into two types of which HIV-type 1 (HIV-1) is the strain responsible for the present worldwide epidemic of AIDS and is the subject of the following studies.

1.1. HIV-associated neurodegenerative disorder

While the primary pathophysiology of HIV is typically associated with immune dysfunction and dysregulation, cognitive impairments have been a long-recognized consequence of infection. The spectrum of progressive neurological complications of infection are characterized into three groups, ranging from asymptomatic neurocognitive impairments (ANI) to mild neurocognitive disorders (MND) and the more severe HIV-associated dementia (HAD) (2). These cognitive dysfunctions, referred to under the umbrella term HIV-associated neurological disorders (HAND) are diagnosed via neuropsychological testing and functional status assessments, and have been reported to affect 20-50% of HIV-infected individuals (2, 3). Since the implementation of combined antiretroviral therapy (cART) as the primary treatment regimen for HIV/AIDS in the mid 1990's there has been a significant reduction in the incidence of the severest form of HAND, now rarely seen in developed countries in the post-cART era. However, prevalence of milder forms of HAND remain stable and are expected to rise with the aging population of HIV-infected individuals (4). In the United States, of the 1.2 million people living with HIV an estimated 50% are older than 50 years of age, with an expected increase to 70% of the population of infected individuals by the year 2030 (5).

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Complications from HIV infection may overlap with other age-associated neurodegenerative diseases such as Alzheimer's disease. Several groups have reported an increased accumulation of amyloid-β precursor protein (APP) in the brains of HIV infected patients (6-8). It is hypothesized that neuroinflammation resulting from the activity of proinflammatory HIV proteins, including Tat and gp120, as well as prolonged cART and aging all contribute to the overall increase in amyloid deposition. Thus, long term infection with HIV might contribute to the worsening of age-related neuronal damage with evidence increasingly suggestive that HIV infection exacerbates age-associated cognitive decline (9, 10). With the shift of HIV infection to a chronic disease in the post-cART era, so too does the potential for significant effects on cognitive decline in the aging population of infected individuals.

HIV invades the central nervous system (CNS) during early infection and viral RNA has been detected in samples of cerebral spinal fluid (CSF) and in the brain within weeks of initial viral exposure with compartmentalized HIV replication detected in the CNS within four months of infection (11). The early infiltration of independently replicating virus within the CNS may represent a mechanism by which the virus establishes a pharmacological and immunological sanctuary site, owing to the inability of most antiretroviral drugs to cross the blood brain barrier and the restricted entry of lymphocyte populations into the brain (12-16). Assessment of cognitive impairment in a population of long-standing aviremic patients yielded an estimated prevalence of HAND in individuals adherent to cART regimens at 69% (17). Indeed, numerous studies support the occurrence of cognitive impairment in HIV-infected individuals despite cART-mediated suppression of HIV viral load in plasma (18-20). HIV RNA viral load in the CSF has been reported to be significantly correlated with neurological dysfunction independent of plasma viral load (21). Thus, evidence increasingly supports the CNS as a site of persistent low-level HIV infection.

HIV infects the CNS via a "Trojan Horse" model of blood-brain barrier (BBB) crossing that is characteristic of lentiviral spread in the bloodstream and CSF (22). HIV infiltrates the brain via infected CD4+ macrophages and lymphocytes, allowing for transmigration of virus to perivascular spaces of the CNS while evading immune detection. Here, the infection is propagated in populations of perivascular macrophages and microglia (23). Infected cells secrete neurotoxic viral proteins, inflammatory cytokines, and small metabolites that may contribute to further disruption of the BBB and promote continued influx of the virus (24, 25).

HIV infection of the CNS is associated with activation of microglia and astrocytes, as well as the induction of inflammatory and neurotoxic insults which contribute to the neurodegeneration and cognitive decline characteristic of HAND. Despite the prevalence of HIV-associated cognitive dysfunctions, the molecular and cellular mechanisms underlying HAND are poorly understood and are thought to consist of a combination of direct viral infection of cells of the CNS and indirect mechanisms involving host factors and neurotoxic effects of HIV-associated proteins.

Notably, HIV viral proteins Tat and gp120 have been implicated in affecting BBB integrity and promoting viral entry into the CNS. Tat (HIV *t*rans*a*ctivator of *t*ranscription), a viral regulatory protein responsible for activating viral transcription, is one of the first HIV proteins to be expressed following infection (26). Tat has been recognized for its neurotoxic role in HAND and similarly to gp120 is found in extracellular spaces in soluble form (27). In the CNS, Tat has demonstrated neurotoxicity, including induction of neuronal oxidative stress via secretion of cytokines and chemokines and neuronal apoptosis via a pathway of NMDA receptor-mediated glutamate excitotoxicity (28, 29). Tat has been detected in postmortem brains and CSF of HIV infected subjects, and numerous studies support Tat-mediated permeabilization of the BBB (30-32). A recent study utilizing a Tat-expressing transgenic murine model to examine the influence of Tat expression on BBB integrity found Tat exposure is sufficient to destabilize BBB integrity and increase the presence of activated perivascular macrophages and microglia in an in vivo model of HAND (33). These results support previous evidence that Tat-dependent disruption to the BBB may also contribute to glial activation and inflammation that underlie indirect neuronal injury observed in HAND (34). While a role for Tat in HAND is clearly supported, Tat's contribution is beyond the scope of this dissertation and is discussed elsewhere in greater detail (35).

Viral surface protein gp120 has also been linked to functional impairment of the BBB. As well as documented gp120 activation of proinflammatory genes and increased migration of monocytes across the BBB, a significant increase in permeability of brain endothelial cells in the presence of circulating gp120 has been reported (36, 37). In both reports, removal of gp120 restored integrity of the BBB. The remainder of this introduction will focus on gp120-mediated neurotoxicity.

1.2 HIV Gp-120

The entry of HIV to host cells is mediated by envelope glycoproteins gp120 and gp41. The non-covalently linked gp120 and gp41 interact to form a trimer of gp120/gp41 heterodimers where gp120 serves as a viral surface protein recognizing host CD4 and chemokine co-receptors and the transmembrane gp41 functions as a fusion peptide to assist in viral-host membrane fusion (38). Given the relatively weak association between the two subunits, gp120 shed from the trimer is well documented (39, 40). Indeed, high levels of monomeric soluble gp120 have been detected in secondary lymphoid organs of chronically infected individuals, while high levels of anti-gp120 antibodies have been detected in cerebral spinal fluid (CSF) of patients with HAD (41-43).

1.2.1 Gp120 interaction with membrane lipid raft domains

Binding of viral surface glycoprotein gp120 to receptors is restricted to host-cell lipid raft domains (Figure 1.1) (44). Lipid rafts are small (10-200nm), dynamic cholesterol and sphingolipid

enriched microdomains in the plasma membrane that serve to compartmentalize cellular processes, facilitating protein-lipid and protein-protein interactions and signal transduction events (45-47). Membrane rafts have been implicated as critical signaling nodes of several neurological pathologies including Alzheimer's and Parkinson's diseases where they appear to play a role in mediating pathologic signal transduction characteristic of these neurodegenerative diseases (48, 49). It has been demonstrated that many pathogens in addition to HIV exploit the lipid raft environment, including influenza and Ebola, at various stages of the viral lifecycle (viral entry, assembly, and budding) (50). The life cycle of HIV is intricately tied to raft domains and requires interaction with raft-localized receptors for initial binding, viral-host membrane fusion, virion assembly, and subsequent release of viral progeny (51-54).

Several studies support the crucial role of rafts for HIV target receptor function. Fluorescence resonance energy transfer imaging has revealed a requirement for lipid rafts in the interaction between CD4 and co-receptors, thus suggesting receptor localization to lipid raft domains (55). CD4 is enriched in lipid raft domains where it serves to facilitate coalescence of these domains and mediates the attachment and entry of HIV into host cells (53, 55, 56). Site-directed mutagenesis has been used to identify a short sequence of positively charged amino acid residues in the cytoplasmic domain of CD4 controlling its targeted partitioning into raft domains (57). In addition to CD4, HIV co-receptors CCR5 and CXCR4 are also found in detergent resistant membrane fractions following exposure to HIV gp120. Host membrane cholesterol is but one particular lipid that is required for HIV infection, as evidenced by the blocking of viral entry following treatment with the cholesterol chelator methyl-β-cyclodextrin. Interestingly, the specific lipid environment of raft domains appears to be necessary for the conformational stability and function of these receptors and putative cholesterol binding sites in both chemokine receptors that appear to support their localization to raft domains have been identified (58). These results support previous studies identifying a requirement for cholesterol in conformational integrity and function of both CCR5 and CXCR4 receptors (52, 59).

1.2.2 Gp120 co-receptor tropism

In most cases, gp120 binding to CD4 alone is not enough to induce membrane fusion, and interaction with a secondary co-receptor is required (60). Gp120/CD4 interaction initiates a series of successive conformational changes in gp120 structure, ultimately exposing the third variable region (V3) loop which greatly enhances the affinity of gp120 to chemokine co-receptors CCR5 or CXCR4. Viral tropism is determined by V3-loop recognition of these co-receptors (61, 62). Notably, viral transmission independent of co-receptor use has been demonstrated on rare occasions, and the establishment of an acute *in vivo* infection by virus with a mutation in the gp120 V3 loop that prevented its binding with CCR5 and/or CXCR4 has been described (63). Evidence of cell-to-cell HIV transmission activated by

contact of infected and uninfected primary $CD4^+$ T cells in the absence of an appropriate co-receptor also has been reported (64). Conversely, in the CNS, gp120 binding to both CCR5 and CXCR4 co-receptors independent of CD4 binding has been documented (65, 66). While these represent instances of CD4/co-receptor-independent mechanisms of HIV transmission, it remains that most productive HIV infections are mediated by gp120 binding to *both* CD4 and co-receptors. Indeed, a "low-CD4" entry phenotype, characterized by gp120 capable of infecting cells expressing low densities of CD4, have been preferentially detected in the CSF of people with HIV-associated dementia. This may be suggestive of viral adaptation to the local cellular environment of the CNS allowing virus to infect a population of cells expressing lower densities of CD4 (67) .

Distinct strains of HIV can be categorized on the basis of cellular tropism tied to co-receptor preferences. Macrophage tropic (R5) strains bind CCR5 receptors and preferentially infect peripheral blood mononuclear cells (PBMC), monocytes, macrophages, and T-lymphocytes, but not T-cell lines. T-cell tropic (X4) strains bind CXCR4 receptors of T-lymphocytes and T-cell lines, and dual-tropic (R5X4) strains bind both CCR5 and CXCR4 receptors (68). Whereas R5 strains are detected throughout all stages of infection and disease, the population of viral strains in an individual often evolves during the course of infection, and an initial predominance of R5 strains gives way to the emergence of R5X4 and X4 strains in an estimated 50% of individuals as infection progresses (69).

The selective pressures driving the switch from CCR5 to CXCR4 receptor usage by HIV are not well understood, though there is evidence to support different hypotheses which may explain the underlying mechanisms. One hypothesis posits that the emergence of CXCR4-binding virus results from a depletion of susceptible CCR5-positive target cells as infection proceeds. This hypothesis is supported by data indicating infected individuals heterozygous for a 32 base-pair deletion in the CCR5 gene, which results in lower expression of CCR5, have higher incidence of X4 viruses when compared to infected individuals with normal CCR5 expression levels (70, 71). Alternatively, a 2016 study provides evidence for host humoral immune pressure selecting against CCR5 variants facilitating the emergence of CXCR4 utilizing virus (72). Although the mechanisms of tropism switch may not be clear, the switch in receptor usage has demonstrable clinical implications and the emergence of X4 strains have been linked to more severe illness and a more rapid progression to AIDS (73).

Feline immunodeficiency virus (FIV) represents an animal model of immunodeficiency with similarities in pathogenesis to HIV infection in humans, including subsequent FIV infection of the CNS in domestic cats that results in neurological symptoms comparable to those observed in HAND (74). A recently published study investigating the role of FIV envelope glycoprotein gp95-mediated synaptic dysfunction found gp95 signaling through neuronal CXCR4 facilitates an elevation in intracellular Ca^{2+} and subsequent increase in synaptic activity (75). Notably, the use of HIV antiretroviral drugs on FIV

infected cats has been demonstrated to significantly reduce viral load, which may be suggestive of a similar mechanism for neuropathogenesis between the two lentiviruses, lending further support to a role for CXCR4 signaling in HIV neurotoxicity (76, 77).

1.2.3 Gp120 neurotropism

Most cell types in the CNS express HIV target receptors CD4, CXCR4, and CCR5, suggesting that virus invading the CNS has the potential to infect many different cell types. CD4 and CXCR4 receptors have been detected on astrocytes, microglial cells, and neurons. CCR5 expression has been predominantly found on astrocytes and microglial cells, with less consistent evidence to support CCR5 expression on neurons.

Immunohistochemistry and flow cytometry have been used to demonstrate expression of CCR5 receptors on neurons of macaques and humans and in particular, on hippocampal neurons of patients with AIDS (71, 72). Notably, neuronal expression of the receptor was decreased in the brains of AIDS patients with HIV encephalitis as compared to AIDS patients without HAND (78, 79). In contrast, immunohistochemical analysis of the expression of CCR5, CXCR4, and several other chemokine receptors in brains of AIDS-positive and AIDS-negative patients found only CXCR4 expression on neurons whereas CCR5 expression was restricted to glial cells (80). These results are in accordance with those of several other studies reporting CCR5 expression is restricted to glial cells of the CNS (81-83). Whether the purported differences in neuronal CCR5 expression are the consequence of differences in experimental approaches or representative of true physiological differences in receptor expression has yet to be determined. There is some indication that expression of chemokine receptors in cells of the CNS is dependent on cell culture conditions and the cytokine environment. Interestingly, a study of CCR5 expression in rhesus macaque brains found expression of receptors on cortical neurons increased with age and demonstrated differential expression in subpopulations of neuronal cells (84, 85). Such dynamic receptor expression may be a factor in the inconsistencies reported above.

Though HIV co-receptors CCR5 and CXCR4 are widely expressed on cells of the CNS, productive infection appears to be restricted to microglia and macrophages, although limited latent infection of astrocytes has been demonstrated (86-89). Productive infection of neurons has been reported but remains rarely observed (90, 91).

1.2.4 Mechanisms of CNS dysfunction

Despite limited evidence supporting direct infection of neurons, it remains that synaptic dysfunction and neuronal cell death are prominent features of HAND and likely underlie the cognitive and motor dysfunctions exhibited by infected individuals (92-94). Many studies investigating the

mechanisms by which HIV impedes CNS function focus largely on neuronal cell death, which is attributed to both direct neurotoxic effects of soluble HIV proteins shed from infected cells, as well as bystander damage The latter is a consequence of activated macrophages, microglia, and astrocytes releasing pro-inflammatory cytokines and chemokines leading to perturbed neuronal homeostasis (95-99). While neuronal loss explains some degree of reported neurological complications of infection, neuronal death alone does not account for the cognitive impairments observed in HAND (100, 101). HAND has been linked to impaired neuronal plasticity, characterized by synaptodendritic damage and decreased synaptic and dendritic density. Given the reduction of the severity of HAND, and in some instances the partial reversal of HAND symptoms, following initiation of cART, it is likely that a loss of synaptic plasticity and synaptodendritic injury account for much of the neuronal pathology observed in the HIV infected brain (102). Indeed, synaptic dysfunction is emerging as an important neuropathologic mechanism underlying early CNS deficits and a role for several HIV proteins has been implicated in synaptic loss (75, 103, 104).

As previously described, FIV envelope protein gp95 has been implicated in CXCR4-mediated synaptic dysregulation. In this model, intracellular Ca^{2+} increase is facilitated by the activation of both endoplasmic reticulum (ER) associated calcium channels and inositol triphosphate receptors (IP3Rs), and similarly to gp120, the activation of synaptic NMDARs. Notably, the neuronal nitric oxide synthase (nNOS)-cGMP pathway was found to be activated by *both* FIV gp95 and HIV gp120 stimulation of NMDARs. Gp95/gp120 interaction with CXCR4 promotes IP3 production and the nNOS induced activation of cGMP-dependent protein kinase II (cGKII). Subsequent phosphorylation of IP3Rs and serine 845 of AMPA receptor subunit GluA1 by cGKII leads to elevated intracellular Ca^{2+} levels and the promotion of AMPA receptor expression at the cell surface resulting in increased synaptic activity and Ca^{2+} hyperexcitation (75).

Gp120 is among the most potent HIV neurotoxins with deleterious effects reported i*n vitro* for concentrations ranging from 1 pM to 1 uM (105, 106). Such potency, in the picomolar and nanomolar range in an *in vitro* model is generally indicative of true *in vivo* biological relevance. There has been some controversy regarding the physiological relevance of gp120 concentrations used *in vitro* to extrapolate its *in vivo* effects. Early attempts to quantify gp120 concentrations *in vivo* relied on capture enzyme-immunoassays which may be influenced by interfering plasma antibodies or limitations in the specificity of the detecting antibody (106-108). While current understanding of *in vivo* concentrations of soluble gp120, particularly in the CNS, remains unclear, there is substantial evidence for a pool of soluble gp120 that has biological activity independent of productive infection (43). Indeed, *in vitro* and *in vivo* studies have demonstrated gp120-mediated cell death in various neuronal populations and the resulting neuronal

and synaptic loss, axonal retraction and dendritic simplification are similar to neuropathy observed in postmortem brains of HAD subjects (109, 110).

1.2.5 Gp120-mediated neuronal apoptosis

Several studies have identified a role for enhanced N-methyl-d-aspartate receptor (NMDAR) activation in gp120-mediated neurotoxicity. NMDA receptors are widely expressed on neurons and enhanced activation of NMDAR by high concentrations of endogenous glutamate results in the rapid influx of Ca^{2+} and free radical generation, ultimately activating pathways leading to neuronal apoptosis (110, 111). Chronic NMDAR mediated excitotoxicity has been implicated in several neurodegenerative diseases including Alzheimer's disease and Huntington's disease and excitotoxicity in HAND has been linked to the gp120-induced release of excitatory molecules, including glutamate, from activated microglia and astrocytes in the CNS (89, 112-114). Interestingly, gp120 has also been shown to act directly on NMDA receptors via interaction of gp120 V3 loop to the glycine site of the receptor and may be indicative of direct mechanisms of neurotoxicity induced by gp120 interaction with NMDA receptors (115).

In addition to NMDAR mediated neurotoxicity, gp120 has been associated with the generation of reactive oxygen species (ROS) and nitrosative stress which are potentially toxic for neurons. Astrocyte exposure to gp120 led to production of ROS and tumor necrosis factor-alpha (TNF-α) in N9 murine microglial cells and stimulated upregulation of inducible nitric oxide synthase (iNOS), an important source of nitric oxide (NO) and nitrosative stress (116, 117). In these indirect models of gp120 neurotoxicity, release of excitotoxic mediators from surrounding macrophages and glial cells induces neuronal apoptosis via disruption of neuronal homeostasis. These results suggest that mitigating oxidative stress in HAND through the use of antioxidants to protect against neuronal apoptosis arising from indirect insults mediated by gp120 might be effective therapy, and several studies have investigated this approach with varying degrees of success (117, 118). Gp120 may also act directly on neurons to enhance NMDA-evoked calcium flux, possibly via modifications in spatial localization and focal density of NMDA receptors to lipid raft domains (119). Gp120 has also been demonstrated to induce activation of nNOS directly in neurons, representing another potential source of NO and nitrosative stress, as well as a possible mediator of calcium hyperactivity and resulting synaptic dysregulation (75, 120).

Notably, neurotoxic effects of gp120 in the absence of NMDAR activation or production of inflammatory cytokines has been demonstrated. In the absence of productive viral infection of neurons, soluble gp120 in the CNS can induce neurotoxicity via direct interaction with chemokine receptors expressed on neuronal surfaces (121). Gp120 induced cell death in human neuroblastoma cells through direct interaction with both neuronal CXCR4 and CCR5 chemokine receptors (122). Further support for

the role of chemokine receptors in mediating gp120 neurotoxicity comes from the discovery that endogenous chemokines are capable of blocking gp120-induced apoptosis of hippocampal neurons (123). Interestingly, while endogenous CCR5 ligands, including macrophage inflammatory protein (MIP)1α, MIP-1β, and 'regulated-and-normal-T cell-expressed and secreted' RANTES have been shown to slow progression to AIDS, the CXCR4 endogenous ligand SDF-1α has been demonstrated to induce neuronal apoptosis in some populations of cells following binding of the CXCR4 receptor (124, 125). It should be noted that the majority of viruses isolated from the CNS are R5 strains, while R5X4 and X4 binding variants occur less frequently (126, 127). Despite this, signaling through CXCR4 has been identified as an important pathway in gp120-induced neuronal apoptosis (2, 128).

1.2.6 Gp120-induced synaptic and dendritic dysfunction

Synaptodendritic damage has emerged as a hallmark of HIV infection of the CNS, and several groups have reported a role for gp120 in synaptic dysfunction and dendritic simplification observed in HAND. An early neuro-histological assessment of synaptic and dendritic markers identified a strong correlation between presynaptic and neocortical dendritic damage and abundance of gp120 in neocortical gray and deep white matter (129). The authors posit that in this model of neurotoxicity, glutamate signaling through NMDA receptors on dendritic membranes is a likely contributor to the observed pathology. Gp120 was similarly found to induce significant loss of synaptic connectivity in hippocampal neurons in the absence of neuronal apoptosis via an indirect mechanism involving the activation of NMDA and CXCR4 receptors, and requiring the release of cytokines from non-neuronal cells (130). Gp120 was further found to induce gliosis and neuronal dendritic injury in a primary mixed human CNS culture analogous to dendritic injuries observed in post-mortem brains of HIV infected individuals (131).

Subsequently, there is evidence suggesting that gp120 neurotoxicity is the result of spatially distinct mechanisms. In an investigation of HIV-associated sensory neuropathy, gp120- mediated axonal degeneration in rat spinal dorsal root ganglia (DRG) occurred as a result of two independent mechanisms (132). Prominent axonal toxicity in the absence of apoptosis was observed only when gp120 was applied directly to sensory axons, with no effect when applied to cell bodies. Conversely, gp120 mediated neuronal apoptosis and subsequent axonal degeneration was found to be dependent on the activation of Schwann cells. Notably, gp120-induced direct axonal degeneration was found to be dependent on the local activation of the caspase pathway and mediated by activation of both CXCR4 and CCR5 receptors (132). Blocking the CXCR4 receptor by monoclonal antibody prevented gp120-induced axonal toxicity and partial prevention of toxicity occurred by blocking the CCR5 receptor. These results highlight an intriguing mechanism by which axonal degeneration following direct gp120 exposure occurs

independently of toxicity in neuronal cell bodies.

1.3 Raft-associated signaling

Membrane lipid raft domains have been demonstrated to be critical sites of interaction between gp120 and chemokine receptors, mediating both internalization of the viral protein and activation of pathways contributing to apoptosis (Figure 1.2) (133, 134). Lipid rafts also appear to be necessary for appropriate receptor conformation to support CXCR4-chemokine binding (135). Gp120 coupled to CXCR4 in primary neurons induces neuronal apoptosis via activation of the sphingomyelin-catabolizing enzyme neutral sphingomyelinase (NSmase) (134). In this pathway, activation of NSmase is regulated by the NADPH-oxidase (NOX2)-mediated production of superoxide radicals in neurons, leading to the increased production of ceramide, an apoptotic second messenger in a number of cell types including glia and neurons. SDF-1 was also found to induce neuronal apoptosis through the same NSmase-mediated pathway, confirming a role for CXCR4 in this pathway of pathologic signal transduction (134).

While overproduction of ceramide has previously been linked to neurotoxicity, ceramide is an important component of cellular lipid raft domains (136). Experiments have demonstrated coalescence of lipid raft domains into larger platforms following ceramide generation by sphingomyelinases (137). Gp120 binding to cellular receptors has been demonstrated to similarly induce lipid raft coalescence, leading to the increased size and stability of raft domains (119). Raft coalescence has been proposed as a mechanism for clustering receptors and components of receptor-activated signaling cascades, and gp120-induced raft coalescence in hippocampal neurons has been found to promote the forward trafficking and surface clustering of NMDA receptors in rafts in a CXCR4 dependent manner (119, 138). Prolonged surface clustering of NMDA receptors in lipid raft domains of neurons may represent an additional mechanism of gp120 mediated neurotoxicity via perturbation of intracellular Ca^{2+} flux or associated signaling events.

Notably, profound changes in lipid rafts are observed for a number of CNS dysfunctions, including Alzheimer's disease, mild cognitive impairments (MCI), CNS aging, and CNS trauma (49, 139-142). Biophysical properties of lipid rafts are imperative for their signaling capacities and even subtle changes in lipid species rapidly affect overall membrane architecture influencing the structure and function of membrane proteins (143-147). Presumably, entire signaling cascades could be silenced due to imposed constraints on lipid-protein and/or protein-protein interactions. Emerging evidence attributes cholesterol a 'chaperone-like' allosteric function in stabilizing structural elements of many membrane proteins through a **C**holesterol **R**ecognition/Interaction **A**mino Acid **C**onsensus sequence or CRAC motif (-L/V-$X_{(1-5)}$-Y/F-$X_{(1-5)}$-R/K-) or its reverse sequence (CARC motif) (148-153). This sequence establishes interaction with the isooctyl tail (A, L, and V), the sterol ring structure (Y, F), and the 3β-OH group (R,K)

of cholesterol. Spatiotemporal alterations of protein function by cholesterol interactions was demonstrated for neurotransmitter receptors (nAchR, 5-HT2B), CXCR4, the β-adrenergic receptor, Na^+K^+-ATPase, and the C99 domain of amyloid-precursor protein (APP) implicated in promoting the amyloidogenic pathway (58, 150, 152). Aberrations in cholesterol homeostasis are commonly associated with degenerative CNS diseases including AD and MCI.

1.4 Cofilin-Actin Rods: A potential mechanism for gp120-mediated synaptic dysfunction

Protein association with lipid rafts as a mechanism for regulating signaling has been seen in a number of neurodegenerative diseases, most notably in Alzheimer's disease, where lipid raft-anchored cellular prion protein PrP^C serves as a receptor for amyloid-β (Aβ) oligomers (154, 155). Previous research has identified a pathway mediated by ROS generation and NOX leading to the formation of cofilin-actin bundles (rods) initiated by the binding of diverse extracellular ligands, including proinflammatory cytokines and Aβ to raft localized receptors (154, 156). Generation of rod-like inclusions is a cellular response to oxidative stress conditions and is characterized by the local dephosphorylation (activation) of cofilin and subsequent assembly of 1:1 cofilin:actin filaments that bundle into rod-like structures containing intermolecular disulfide cross-linked cofilin (157). Rod formation may be a transient response to oxidative stress and rods can dissociate following relief from oxidative stress. Rod formation might also be responsible for the loss of synaptic function and decreased network connectivity observed in many neurodegenerative conditions including HAND (158, 159). Although actin rod formation has not been identified in FIV *in vitro* or *in vivo*, considerable disruption to the neuronal cytoskeleton has been demonstrated both for filamentous actin structures as well as microtubule structures (161).

Indeed, cofilin sequestration into rods might have a neuroprotective effect due to its ability to promote mitochondrial-dependent apoptosis through its oxidation to form intramolecular disulfide bonds and its ability to target the tumor suppressor protein p53 to the nucleus and mitochondrion (160, 161). However, persistent rods have been described in neurons during the progression of Alzheimer's and other neurodegenerative disease (162). Cofilin-actin rods have also been observed in brains of AD model mice where their decreased abundance through inhibition of cofilin dephosphorylation or reduced cofilin expression results in normalizing cognitive function and long-term potentiation in brain slice cultures (163, 164). In addition to synapse loss due to interrupted vesicular transport resulting from occlusion of neurites in which rods form, cofilin-actin rods have been linked to synaptic dysfunction via sequestration of cofilin from dendritic spines where it plays an important role in post-synaptic plasticity associated with learning and memory (165, 166). Normalization of cofilin activity in dendritic spines through modulation of upstream kinases and phosphatases by cell penetrating peptides has proven to be effective in many

different neurological disorders, including AD (167). Aβ induction of cofilin-actin rods requires the expression of cellular prion protein PrP^C in raft domains and is mediated by ROS generation by NOX (154).

A number of intriguing commonalities in the neuronal response to HIV gp120 stimulation and that of Alzheimer's associated protein Aβ have been identified, among them the enhanced lipid raft coalescence into stable macro domains, the activation of NMDA receptors localized to lipid rafts, and the NOX2 mediated generation of ROS (49, 119, 134). Given these commonalities, it is quite likely that gp120 mediated stimulation of NOX2 also leads to the downstream formation of cofilin rods, thereby inducing synaptic loss and impaired synaptic function similar to what occurs in other neurodegenerative diseases such as Alzheimer's. Interestingly, gp120-mediated CXCR4 signaling has been demonstrated to activate cofilin in resting T-cells as a mechanism of facilitating nuclear localization and replication of the virus (168). Rods have also been demonstrated to be induced by nitric oxide, a source of oxidative stress shown to be generated by both HIV gp120 and FIV gp95 via CXCR4 mediated signaling (75). Further supporting the role of cofilin-actin rods in HIV-mediated synaptic dysfunction, data generated in our lab has shown gp120 is capable of inducing rod formation in rat E18 hippocampal neurons via CXCR4-associated signaling. Similarly to rod induction by Aβ, this pathway appears to be mediated by the presence of PrP^C in membrane raft domains (Smith et al., in preparation.).

Demonstrating the pivotal role of cholesterol as a "chaperone" allosteric modulator of NOX2-dependent superoxide generation would considerably advance our understanding of how membrane architecture regulates protein structure and function, potentially identifying putative interaction surfaces of NOX2 with cholesterol possibly through CRAC/CARC motifs and provide detailed insight into structure/function aspects of NOX2. Exploiting these findings could open new avenues for drug-discovery approaches to target NOX as well as novel therapeutic strategies to ameliorate gp120-induced oxidative stress in the CNS. Entire signaling networks could presumably be silenced by interference with annular lipids, boundary lipids, activating lipids, and/or "chaperone" lipids (146, 147, 169-171).

1.5 Research objectives

Despite the reduction in incidence of severe forms of HAND, prevalence of milder forms is expected to increase with the aging population of HIV-infected individuals independent of adherence to cART, representing a significant public health challenge. Gp120-associated receptor binding and activation of NOX2-mediated oxidative stress has identified a potential mechanism linking gp120-CXCR4/CCR5-induced ROS formation to pathways of early synaptic dysfunction characteristic of HAND and many other neurodegenerative diseases including Alzheimer's and Parkinson's diseases. The main objective of this dissertation is to determine the role of gp120 and co-receptor tropism in the

induction of pathologic neuronal cofilin-actin rods in three model systems: rodent derived primary neurons, human neuroblastoma cells, and human embryonic stem cell derived neurons. The research approach focused on the following aims:

1.5.1 Aim 1: What are the consequences of distinct gp120 co-receptor tropism on the generation of rod-like cofilin-actin inclusions in primary rodent neurons (Figure 1.3)?

Preliminary experiments in our lab revealed dual tropic gp120 induced the formation of cofilin-actin rod like structures in distal neurites of primary rodent neurons. This study aimed to further delineate the roles of the individual chemokine co-receptors CXCR4 and CCR5 in the generation of rods and their part in the activation of a previously described molecular pathway leading to rod formation. These studies identified a molecular pathway leading to neuronal rod formation requiring both active NOX and expression of the cellular prion protein (PrP^c) that is initiated by the binding of a number of extracellular ligands, including the Alzheimer's associated Aβ oligomers and proinflammatory cytokines. Given that Alzheimer's disease and HAND are both characterized by oxidative stress and neuroinflammation, we tested the hypothesis that gp120 binding to chemokine co-receptors CXCR4 and/or CCR5 induces formation of rods in a NOX-PrP^c dependent manner by exposing E16.5 mouse hippocampal neurons to three different gp120 strains— dual-tropic, R5-tropic, and X4-tropic gp120. The results from this study provide evidence for gp120 induced actin-cofilin rod formation through the PrP^c/NOX2 signaling pathway as a previously unknown mechanism of early synaptic dysfunction in HAND.

1.5.2 Aim 2: Does gp120 stimulate lipid raft coalescence, NOX-mediated generation of ROS, and cofilin-actin rod formation in SH-SY5Y human neuroblastoma cells (Figure 1.4)?

Current *in vivo* models for investigating rod formation rely on the use of murine primary neurons, owing to the expense and ethical issues involved in the use of primary neurons of human origin. Neuronal models of HAND also frequently rely on the use of cells of nonhuman origins. As HIV naturally infects only humans, we sought to investigate the role of gp120 in the activation of lipid raft coalescence and induction of oxidative stress and rod formation in a more physiologically relevant model system. Utilizing SH-SY5Y human neuroblastoma cells we assessed the effects of gp120 exposure on undifferentiated and differentiated cells. Given an absence of distinct neuronal processes, undifferentiated SH-SY5Y cells cannot be used to evaluate rod formation downstream of gp120/receptor interactions. However, SH-SY5Y cells can be terminally differentiated to express long, branched neuronal processes and markers of mature neurons. In this study, SH-SY5Y cells were differentiated to examine gp120/co-receptor mediated rod formation. The results of this study found that gp120-induces lipid raft coalescence and the NOX-

mediated generation of ROS, providing evidence for gp120-mediated neurotoxicity. Differentiated SH-SY5Y cells were further evaluated as a non-murine model for rod formation.

1.5.3 Aim 3: Can mature human neurons derived from embryonic stem cell (hESc) serve as a more relevant model for the investigation of gp120 induced actin-cofilin rods (Figure 1.5)?

While SH-SY5Y cells are a widely used model in the investigation of HAND, results obtained from the use of immortalized cell lines may not be necessarily representative of primary human neurons. These immortalized cells often exhibit intracellular regulations that differ from primary human neurons and may have accumulated mutations as a consequence of numerous replications. Previous studies demonstrate that the responses of primary neurons and immortalized neuronal cultures differ in their sensitivities to toxins and the exhibit differential expression of membrane receptors (172, 173). To overcome these limitations and move closer towards a truly physiologically relevant model for the investigation of HAND, we sought to establish a protocol for the direct induction of mature, cortical human neurons derived from hESc and evaluate these induced neurons for gp120 mediated rod formation. The results from this study will provide the basis of future studies to investigate the role of gp120 and co-receptors in HAND in a primary neuronal population of human origin.

1.6. Figures and Tables

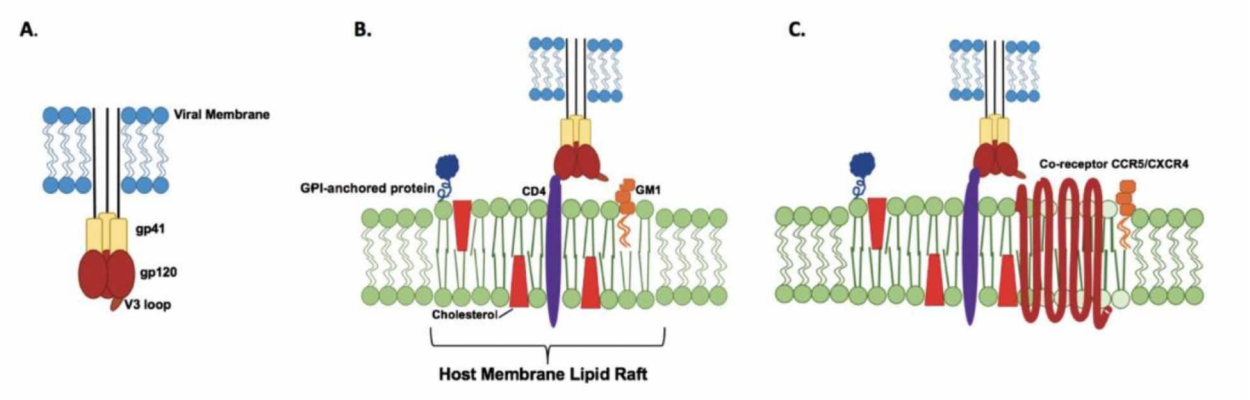

Figure 1.1. Schematic representation of HIV-1 surface proteins gp120 binding to host cell receptors in lipid raft domains. A. Viral membrane bound gp41/gp120 trimers. B. Binding to cellular CD4 receptor facilitates a conformational change in gp120 variable loop 3, allowing for further interaction with chemokine co-receptors CCr5 or CXCR4 (C).

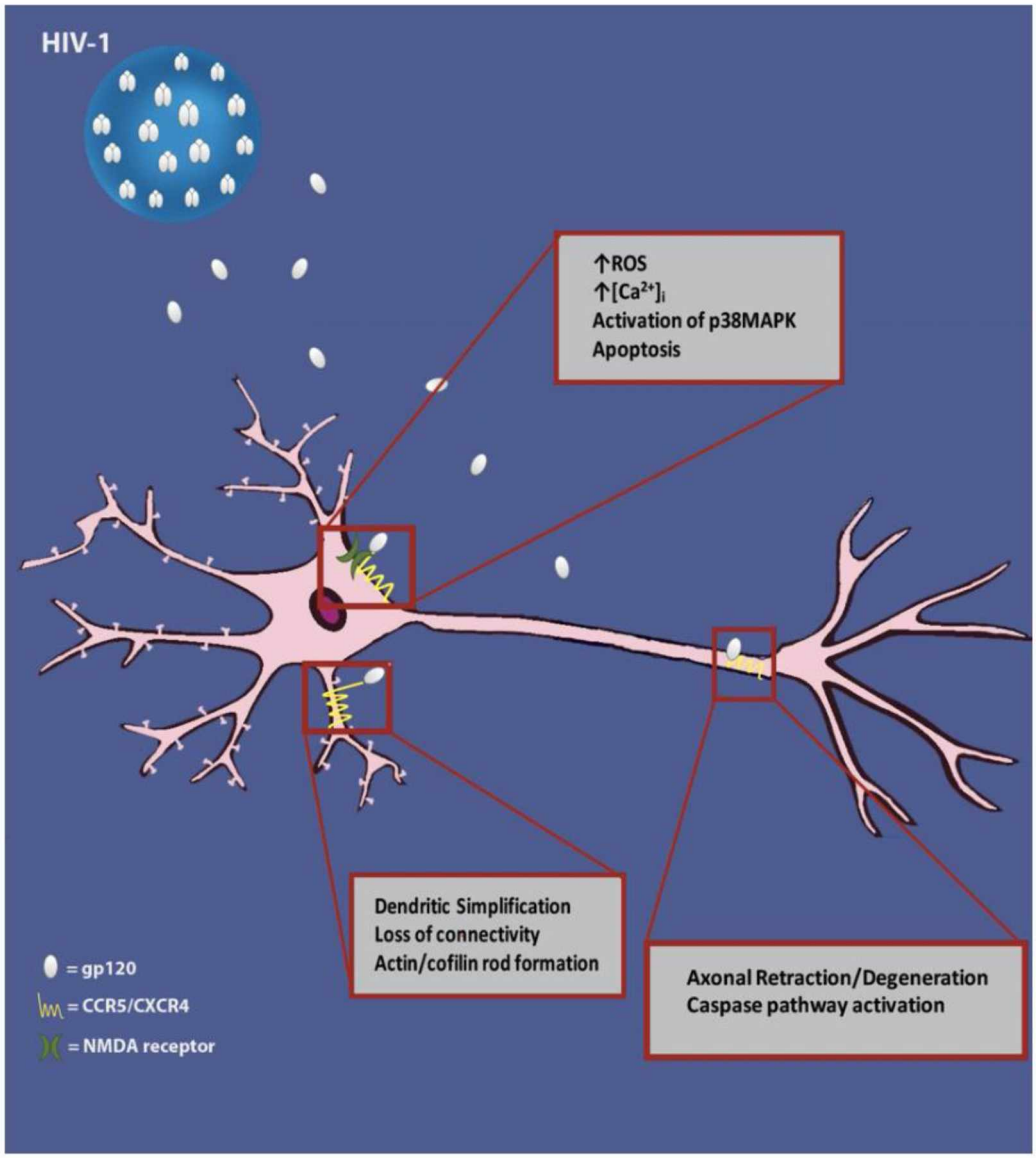

Figure 1.2. Schematic diagram illustrating neurotoxic effects of soluble gp120 interactions with neuronal receptors. Direct interaction of gp120 with both chemokine co-receptors CCR5 and CXCR45, as well as N-methyl-D-aspartate (NMDA) receptors on neuronal surfaces leads to increased generation of reactive oxygen species and intracellular calcium influx, and the activation of signaling pathways leading to cellular apoptosis. In the absence of apoptosis, gp120 has been demonstrated to induce dendritic and axonal degeneration leading to a loss of neuronal connectivity and formation of actin-cofilin rods.

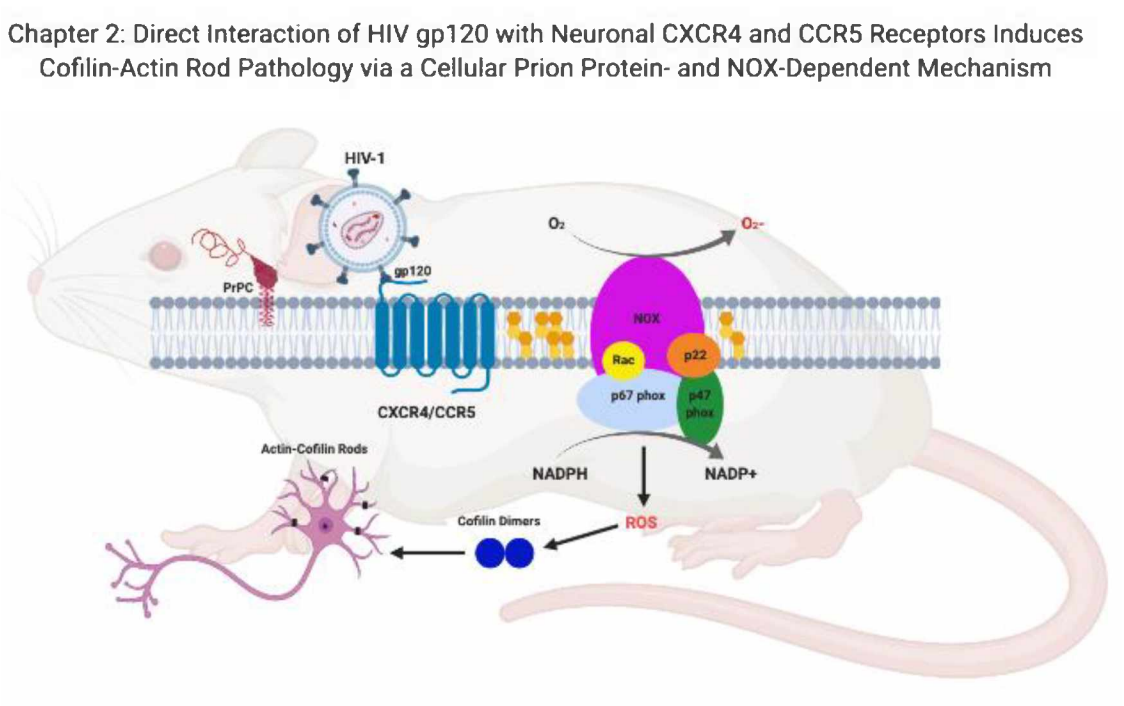

Figure 1.3. Dissertation Chapter 2 Graphical Abstract

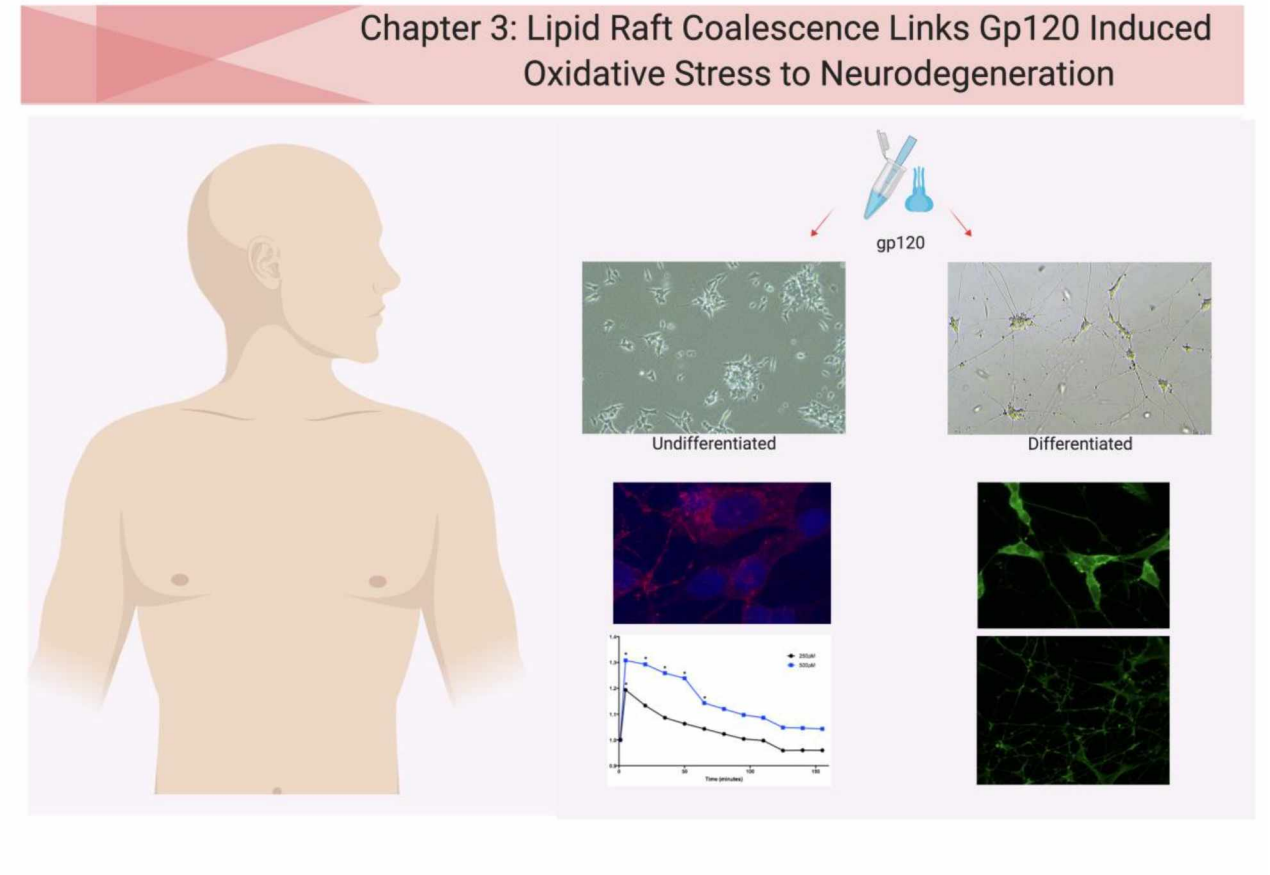

Figure 1.4. Dissertation Chapter 3 Graphical Abstract

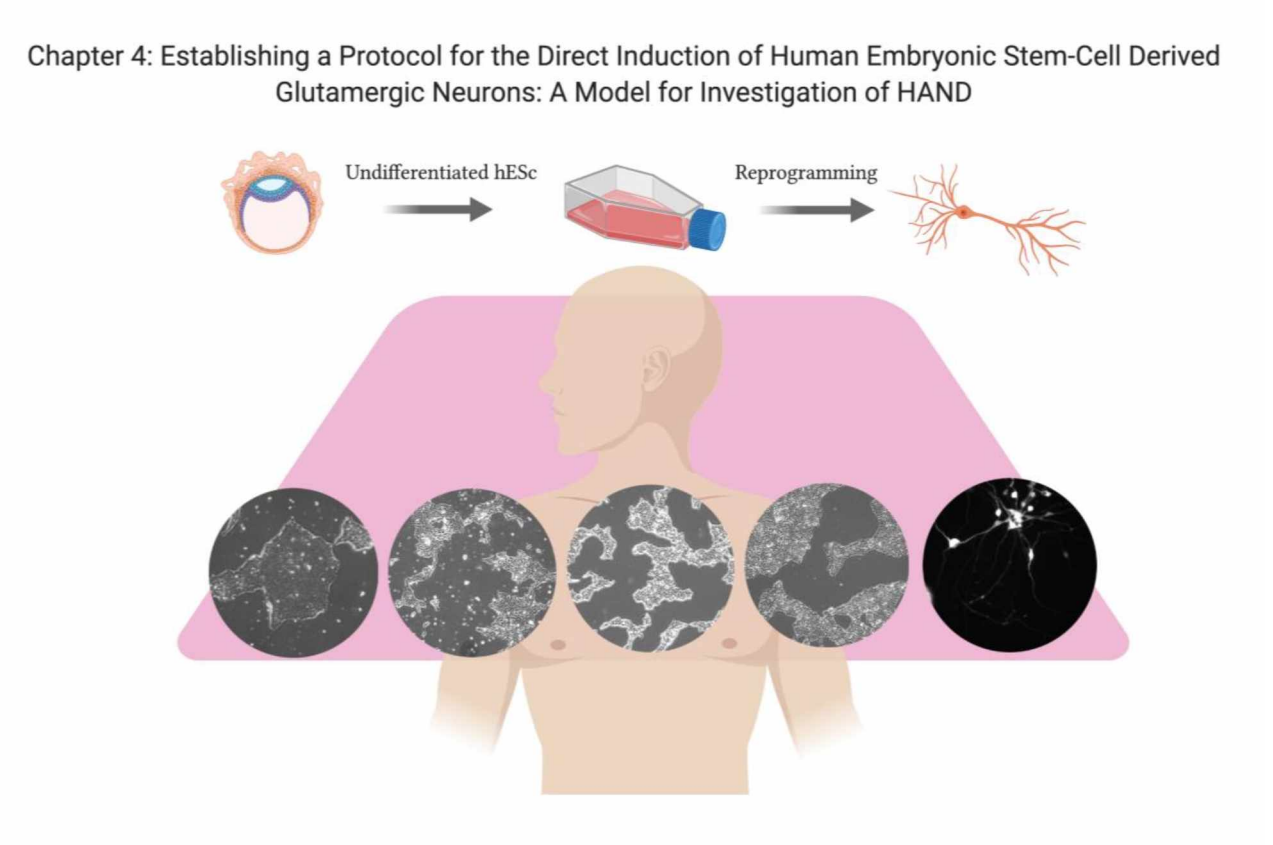

Figure 1.5. Dissertation Chapter 4 Graphical Abstract

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Chapter 2: Direct Interaction of HIV gp120 with Neuronal CXCR4 and CCR5 Receptors Induces Cofilin Actin Rod Pathology via a Cellular Prion Protein- and NOX2-Dependent Mechanism

2.1 Abstract

Nearly 50% of individuals with long-term HIV infection are affected by the onset of progressive HIV-associated neurocognitive disorders (HAND). HIV infiltrates the central nervous system (CNS) early during primary infection where it establishes persistent infection in microglia (resident macrophages) and astrocytes that in turn release inflammatory cytokines, small neurotoxic mediators, and viral proteins. While the molecular mechanisms underlying pathology in HAND remain poorly understood, synaptodendritic damage has emerged as a hallmark of HIV infection of the CNS. Here, we report that the viral envelope glycoprotein gp120 induces the formation of aberrant, rod-shaped cofilin-actin inclusions (rods) in E16.5 mouse hippocampal neurons via a signaling pathway common to other neurodegenerative stimuli including oligomeric, soluble amyloid-β and proinflammatory cytokines. Synaptic function is impaired preferentially in the distal proximity of rods within dendrites. Our studies demonstrate gp120 binding to either chemokine co-receptor CCR5 or CXCR4 is capable of inducing rod formation and signaling through this pathway requires active NADPH oxidase, presumably through the formation of superoxide (O_{2^-}) and the expression of cellular prion protein (PrP^C). These findings link gp120-mediated oxidative stress to the generation of rods, which may underly early synaptic dysfunction observed in HAND.

2.2 Introduction

HIV infection of the CNS is characterized by the induction of inflammatory and neurotoxic insults, including the activation of microglia and astrocytes, suspected to stimulate a progressive synaptic degeneration manifested in cognitive decline. Despite the prevalence of HIV-associated neurocognitive disorders (HAND) the underlying molecular and cellular mechanisms promoting pathogenesis remain poorly understood but are thought to consist of a combination of direct viral infection of the cells of the CNS and indirect neurotoxicity mediated by inflammatory cytokines, metabolites, and effects of viral proteins including the envelope glycoprotein gp120. Gp120 is a potent neurotoxin with roles in a number of indirect and direct neurotoxic pathways including the release of excitatory molecules, reactive oxygen species (ROS), and pro-inflammatory cytokines from activated microglia and astrocytes (indirect) as well

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as NMDAR mediated excitotoxicity and co-receptor mediated neuronal apoptosis arising from the direct interaction of gp120 with receptors expressed on the neuronal membrane (Lipton et al., 1991; Bell, 1998; Kaul et al., 2001; Chen et al., 2002; Wang et al., 2003; Lewerenz and Maher, 2015).

Gp120 facilitates viral entry to host cells via its interaction with primary host-cell receptor CD4 and chemokine co-receptors CCR5 and CXCR4 at host-cell lipid raft domains (Kozak et al., 2002). Gp120 co-receptor preference categorizes distinct strains of HIV on the basis of cellular tropism, with macrophage tropic (R5) strains binding CCR5 receptors, T-cell tropic (X4) strains preferentially binding CXCR4 receptors, and dual-tropic (R5/X4) strains binding both co-receptors (Wilen et al., 2012). Binding of gp120 to cellular receptors induces coalescence of lipid raft domains into large, stable platforms— a proposed mechanism for clustering receptors and components of receptor-activated signaling cascades observed in a number of CNS dysfunctions, including CNS aging and trauma, as well as Alzheimer's disease (Jana and Pahan, 2004). Indeed, gp120 was found to enlarge and stabilize raft domains in a CXCR4-dependent pathway involving the redox-sensitive translocation of neutralsphingomyelinase 2 (nSmase2) to the membrane and the forward trafficking, surface expression, and clustering of NMDA receptors to enlarged raft domains (Cremesti et al., 2002; Jana and Pahan, 2004). These studies are consistent with macrodomain formation promoted by the release of ceramide from nSmase2-mediated hydrolysis of sphingomyelin to activate signaling in response to various agonists and stress signals. Specifically, a redox-sensitive translocation of nSmase 2 is mediated by the gp120 stimulating a lipid-raft localized NADPH-oxidase 2 (NOX2) with a subsequent production of superoxide (O_{2^-}) radicals in neurons (Jana and Pahan, 2004).

The interaction of proteins with lipid-raft localized receptors as a mechanism of regulating pathologic signaling has been observed for a number of neurodegenerative diseases, most notably in Alzheimer's disease where soluble, stable amyloid-β dimers and trimers ($A\beta_{d/t}$) interact with the lipid raft-anchored cellular prion protein PrP^C to stimulate a pathway mediated by activated NOX leading to the formation of rod-shaped bundles of filaments composed of a 1:1 complex of cofilin-actin (Iskander et al., 2004; Bamburg et al., 2010; Minamide et al., 2010; Walsh et al., 2014). These rod-like inclusions are generated in response to oxidative stress conditions and arise from the oxidation of active (dephospho) cofilin in stressed neurons to form intermolecular disulfide cross-linked cofilin (Bernstein et al., 2012). Rods have been described during the progression of Alzheimer's and other neurodegenerative diseases where they contribute to cytoskeletal abnormalities and synaptic dysfunction through the disruption of normal actin dynamic, the blocking of neuronal transport, and the sequestration of cofilin from dendritic spines resulting in dendritic simplification (Minamide et al., 2000a; Maloney et al., 2005; Gu et al., 2010; Cichon et al., 2012; Rahman et al., 2014).

Given the similarities in the neuronal response to HIV gp120 and that of $A\beta_{d/t}$ it is feasible that gp120-sensitive production of O_{2^-} mediated by NOX2 is similarly inducing the downstream formation of cofilin-actin rods. Here, we provide evidence that gp120 signaling through chemokine co-receptors CCR5 and CXCR4 induces the formation of cofilin-actin rods via a pathway common to $A\beta_{d/t}$ and proinflammatory cytokines comprised of PrP^C and NOX2.

2.3. Methods

2.3.1. Ethics Statement

All animals were handled according to the guidelines provided by the National Research Council for the Care and Use of Laboratory Animals as approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC approved protocol #17-7411A).

2.3.2. Reagents

All chemical reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless indicated otherwise. Tissue culture reagents and immunocytochemistry reagents were from ThermoFisher (Waltham, MA). Primary antibodies used are: rabbit anti-cofilin antibody (2 ng/mL, affinity purified)(rabbit 1439, (Shaw et al., 2004); mouse monoclonal actin antibody (C4; XXXXX); rabbit anti-CXCR4 (NIH AIDS Reagent Database, Catalogue #11232; 1:250 dilution); rabbit anti-CCR5 (NIH AIDS Reagent Database, Catalogue #11236),; rabbit monoclonal antibody to microglia (Iba-1, Abcam 178846) a generous gift from Dr. R.A. Swanson, UCSF; and mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) (Fisher Sci., MA5-15086). Dual-tropic $gp120_{MN}$ and X4-tropic $gp120_{IIIB}$ were obtained from Immunodx (Woburn, MA). For R5-tropic strains $gp120_{CM}$ was obtained from ProSpec Proteins (Ness-Ziona, Israel) and $gp120_{BaL}$ was obtained from NIH AIDS Reagent Database (Catalogue #4961).

2.3.3. Source of neurons

Mouse neurons were obtained from either wildtype C57BL/6, PrP^C null (C57BL/6J-Prnp−/−; Talen) or $p47^{PHOX}$ null (B6N.129S2-Ncf1tm1Shl/J p47phox -/-; JAX 027331) lines. Rat neurons were from Sprague-Dawley rats.

2.3.4. Neuronal cell culture

Hippocampal and cortical neuron cultures were prepared either from freshly dissected from E16.5 fetal mouse or E18 fetal rat brains according to the method of Barlett and Banker or from cell stocks of these dissociated neurons frozen at 10_6 cells/ml (hippocampal neurons) or 10_7 cells/ml (cortical neurons) in 50% Fetal bovine serum (FBS) and self-made Neurobasal medium (sfNB, see below). Briefly, round

glass cover slips (12 mm diameter, #1 German, Carolina Biologicals Supply Co.) inserted into 24 well plates were coated with 100 mg/ml poly-D-lysine (30 min, RT), washed 3 times with ultrapure water, and air. Dissociated neurons were plated at a density of 40,000 neurons per well (0.5 ml medium per well) in 10% FBS (Hyclone, VWR Radnor, PA)-supplemented complete growth medium composed of self-made Neurobasal (sfNB) (made from all component of commercial NB but with highly purified L-serine and adjusted to final concentrations of 175 µM L-cysteine, 2.5 mM glucose and to 300 mOsM with NaCl), Glutamax used at 25 µl/10 ml, 50 U/ml penicillin, 50ug/ml streptomycin, N21-MAX (R&D Systems) at 1ml/50ml. Cultures are incubated at 37°C in a humidified incubator with 5% CO_2). After 1 to 2 h, serum-containing medium was removed, replaced with complete growth medium (1 ml/well) and exchanged every 3 days.

2.3.5. Adenovirus preparation

Recombinant, replication deficient adenovirus for the expression of EGFP-PrP_C, lacZ-GFP, and $DNp22^{PHOX}$ (a dominant negative from of the small membrane NOX subunit $p22^{PHOX}$) were made using the AdEasy system (He et al., 1998; Minamide et al., 2003). Virus titer was determined by immunostaining against E2A of infected HEK293 cells as previously described (Minamide et al., 2003). Titers were expressed as focus-forming units (ffu/ml) and were usually about 10^9 ffu/ml. Recombinant adenoviruses were stored at -80°C.

2.3.6. Adenovirus infection of neurons

Replication deficient, recombinant adenovirus was added to neurons 4 days after plating (days in vitro, DIV) at 30 to 200 multiplicity-of-infection (MOI) to express either EGPF-PrP_C, lacZ-GFP, or $DNp22^{PHOX}$. Infection was executed concomitant with a full medium exchange.

2.3.7. Rod induction in neuronal cultures

Rod induction was initiated at 6 DIV over a time period of 16 h unless indicated otherwise. After a complete medium exchange (1ml/well), doses between 250 pM and 750 pM of dual tropic $gp120^{MN}$, monotropic X4 ($gp120^{IIIB}$) or monotropic R5 ($gp120^{Bal}$ or $gp120^{CM}$) were added in serum-free sfNB. Amyloid beta dimer/trimers ($A\beta_{d/t}$) were isolated from medium of 7PA2 cells as previously described (Walsh et al., 2002; (Cleary et al., 2005; Davis et al., 2011) and used at approximately 1 nM final concentration (monomer equivalent) determined from Western blots with monomeric synthetic Aβ standards.

2.3.8. Culture treatments

Maraviroc (R5 inhibitor, 100 nM, Santa Cruz Biotechnology), AMD3100 (X4 inhibitor, 50 nM, Santa Cruz Biotechnology.) or TG6-227 (NOX inhibitor, kindly provided by Dr. Lambeth) were added to some cultures 1 h prior to addition of gp120 and maintained for the duration of the experiment.

2.3.9. Immunolabeling of rods and chemokine receptors

Following treatments, neuronal cultures were fixed in 4% formaldehyde, 0.1% glutarladehyde in warm (37$_o$C at addition) phosphate buffered saline (PBS) for 30 min at room temperature. Cultures were washed 3 times with PBS and permeabilized with methanol (chilled to -20$_o$C) for 3 min. After several washed with Tris buffered saline (TBS), cultures were treated with blocking buffer (5% goat serum, 1% BSA in TBS) for 1 h prior to the addition of primary antibodies overnight (4$_o$C). After 3 wash steps with TBS, Alexa-488 or Alexa-564 secondary antibodies (1:1000) were applied for 1 h (room temperature), followed by 3 washes in TBS, and coverslips were then mounted onto glass slides with ProLong Gold Antifade containing DAPI (Molecular Probes, Eugene OR).

2.3.10. Rod quantification

Immunolabeled neurons were imaged on an inverted fluorescence microscope using a 40x air objective and scored by an individual blinded to the treatments or by more than one individual. For most experiments, at least 100 neurons per coverslip were scored for the presence of rods with triplicates for each condition and three independent experiments. Neuronal processes in the vicinity of non-neuronal cells and rod-like staining in growth cones were disregarded in the analysis. Scoring in low density cultures was performed by calculating the percent of isolated neurons with rods, whereas in higher density cultures, rod index was scored by counting the number of rods per total nuclei (DAPI) per field of view.

2.3.11. Statistical analysis

Quantification of all experiments were performed with at least triplicate samples for each condition and repeated in at least three independent experiments. For rod analysis, rod index (number of rods per neurons in a field of view) and percent neurons with rods were calculated and independent group averages (from triplicates) were used to calculate the standard deviations shown on plots. Significant differences among treatments and between treatments and controls were tested by one-way ANOVA with Tukey's or Dunnett's posthoc-analysis using Graph Pad Prism software (GraphPad Software, Inc.). An alpha level of 0.05 was used for all statistical analyses.

2.4. Results

2.4.1. Gp120 interacts with neurons to induce actin-cofilin rods in a dose- and time-dependent manner

Prior to experiments examining gp120-mediated rod induction, we first addressed the issue of spontaneous rod formation in neuronal cultures. Through many years of experiments scoring rods in neuronal cultures, untreated controls generally had fewer than 5% of neurons with "spontaneous" rods (e.g. Walsh et al., 2014), in recent years this number became much more variable and often exceeded 25%, with rods/cell (rod index) of 0.5 to 1.0, totally unacceptable in our experiments. As the increase in spontaneous rods is likely due to culture stress, we sought to improve culture conditions to reduce spontaneous rods. By using a complete growth medium based on self-made neurobasal (sfNB) media made with L-serine purchased from a supplier with low D-serine contamination, low glucose (2.5mM) and a physiological osmolarity of 300 mOsM, we reduced sponataneous rods from what we were getting with commercial NB to 5% or less and lowered the rod index to less than 0.2 rods/neuron. Neuronal cultures were maintained in complete growth medium utilizing sfNB as a basal medium for all experiments reported here, with the exception of those examining the role of NOX activity.

Gp120 has been previously demonstrated to be a potent generator of ROS (Walsh et al., 2004; Guo et al., 2013; Louboutin and Strayer, 2014). Given the requirement of oxidized cofilin for the formation of cofilin:actin rods, we examined whether gp120 is capable of inducing rod formation in cultured mouse hippocampal neurons. Neurons at DIV 6 were treated with increasing concentrations of dual-tropic $gp120_{MN}$ for 18 hours before being fixed, immunostained for actin and/or cofilin, and evaluated for rod formation (Figure 1A). For each concentration tested, dual-tropic gp120 induced rod formation significantly ($p<.01$) above the untreated control in a dose-dependent manner (Figure 1B). As dual-tropic gp120 is capable of binding to both R5 and X4 receptors, we further evaluated the ability of mono-tropic gp120 strains to induce rod formation. Neurons exposed to increasing concentrations of X4- and R5-tropic gp120 ($gp120_{IIIB}$ and $gp120_{CM}$) for 18 hours demonstrated rod formation significantly above control at concentrations of 500 and 750 pM for both strains tested (p£0.03) suggesting that both co-receptors are capable of initiating gp120-mediated rod formation (Figure 1B). We next examined the time course of dual-tropic gp120 mediated rod formation. Mouse hippocampal neurons were treated with 500 pM dual-tropic gp120 and the percent of neurons forming rods increased over time, reaching significance above control by 6 h (p£0.003) and half-maximum rod induction at 8 to 9 h (Figure 1C). Previous studies have demonstrated that almost all neurons can form rods when subjected to energy depletion ((Minamide et al., 2000a) but rods are induced in a maximum of 20-25% of cultured mouse hippocampal neurons by $A\beta_{d/t}$ and the proinflammatory cytokine TNFα when used separately or in combination (Walsh et al., 2014), suggesting that both inducers are eliciting rod formation through the same pathway (Walsh et al., 2014).

To determine if dual-tropic gp120 is also inducing rods through the same pathway, mouse hippocampal neurons were treated with 500 pM dual tropic gp120 alone or in conjunction with either 50 ng/ml TNFα or 1 nM $A\beta_{d/t}$ for 24 h before immunostaining and rod quantification. Rods were observed in 20-25% of dual-tropic gp120-treated neurons alone or in combination with $A\beta_{d/t}$ or TNFα (Figure 1D). Interestingly, we also found dual-tropic gp120-mediated rod formation to be reversible as washing out gp120 after a 22 h exposure significantly reduced the percentage of neurons with rods detected 2-hours post washout ($p<.001$) (Figure 1D), similar to what was observed for TNFα-induced rods (Walsh et al., 2014). We observe similar rod induction by gp120 in mouse cortical neurons (dual-tropic gp120), as well as a time-dependent formation of rods in rat cortical neurons with R5 or X4-tropic gp120 (Supplemental Figure S2). Notably, in rat cortical neurons, gp120 signaling through CXCR4 appears to be a more potent inducer of rod formation. Taken together, these findings suggest that gp120, $A\beta_{d/t}$, and TNFα all induce rod formation in a subpopulation of hippocampal neurons through what is likely a common pathway, and that continuous exposure to gp120 is required for the persistence of gp120-induced rods.

2.4.2. Mouse hippocampal neurons express CCR5 and CXCR4 chemokine receptors (R5 and X4)

We next assessed the role of individual co-receptors in the induction of rods. Though most cell types in the human CNS express either chemokine receptor, there has been less consistent evidence of R5 expression on neuronal cells (Bell, 1998; Gabuzda and Wang, 2000; Canto-Nogues et al., 2005; Sorce et al., 2011). To confirm that both X4 and R5 are indeed expressed in rodent hippocampal neurons, both mouse and rat neurons were cultured to DIV7, fixed, and immunostained for each receptor. Both receptors are widely expressed in rodent hippocampal neurons on the soma as well as on all neurites (Figure 2A). As levels of R5 expression in mouse neurons have been reported to increase following exposure to extrinsic stress insults (Joy et al., 2019), we tested whether gp120 exposure may also upregulate R5 membrane expression. Mouse hippocampal neurons were treated with 250 pM of R5 and X4-tropic gp120 for 16 h before being fixed and immunostained for R5 or X4. However, no increase in expression of either receptor was detected (data not shown).

2.4.3. Inhibition of X4 with receptor-specific antagonist AMD3100 blocks rod formation

Whereas gp120 binding to R5 and X4 co-receptors is required for facilitating viral envelope fusion with the host membrane, interactions with other neuronal receptors, including N-methyl-D-aspartate receptors (NMDAR) and nicotinic acetylcholine receptors (nAChR) have been reported (Bracci et al., 1992; Xu et al., 2011; Ballester et al., 2012; Capó-Vélez et al., 2018). Having demonstrated that gp120 co-receptors are present in the membrane of rodent hippocampal neurons, we sought to confirm that it is the interaction of gp120 with these specific chemokine receptors that triggers rod formation. To

this end, we exposed 6 DIV cultured mouse hippocampal neurons to 250 pM R5, X4, or dual tropic gp120 in the presence of 100 nM maraviroc and 50 nM AMD3100, receptor specific inhibitors for R5 and X4, respectively. In neurons exposed to either R5-tropic gp120, we did not measure a significant decrease in rod index with the inhibition of either receptor although there was a measurable decrease in rod index in cultures exposed to maraviroc (Figure 2B). Rod index was restored to control levels in neurons exposed to X4-tropic gp120 in the presence of AMD3100, suggesting that gp120-mediated signaling through X4 may be a more potent activator of rod induction (Figure 2B). Neither maraviroc nor AMD3100 significantly affected rod induction in cultures exposed to the opposite receptor-binding gp120 strain. For dual-tropic gp120, only AMD3100 significantly reduced the number of rods, confirming the observation that rod induction is more sensitive to gp120 signaling through X4 (Figure 2B). Indeed, for both dual- and X4-tropic gp120, blocking X4 with AMD3100 returned rods to basal levels supporting the hypothesis that rod induction by gp120 is occurring through a CXCR4-mediated signal transduction pathway.

2.4.4. Gp120 mediates rod formation through a cellular prion protein PrP^C- dependent pathway that requires the NOX activation

Rod induction by $A\beta_{d/t}$ and proinflammatory cytokines occurs through a PrP^c-dependent signaling pathway linking these ligands to the activation of NOX (Walsh et al., 2014). We hypothesized that gp120-mediated rod induction must also require membrane expression of PrP^C and active NOX. To test this hypothesis, we exposed hippocampal neurons cultured from PrP^C null mice to all three gp120 tropic strains. Unsurprisingly, none of the tested strains of gp120 induced rod formation (Figure 3A). To verify the requirement of PrP^C expression for gp120 mediated rod formation, we infected PrP^C-null hippocampal neurons with adenovirus expressing EGFP-PrP^C driven by a cytomegalovirus (CMV) promotor that has previously been demonstrated to drive the expression of functional PrP^C at the membrane surface (Haigh et al., 2005). In neurons re-expressing PrP^C, 18 h treatment with 250 pM of each tropic strain of gp120 induced a significant increase in rod formation over control (p£0.005) (Figure 3B). Having confirmed the essential role of PrP^C in mediating gp120 induced rod formation, we next sought to confirm the requirement for active NOX.

Active NOX2, a superoxide (O_2^-) generating multi-subunit enzyme, is comprised of two membrane subunits ($gp91^{PHOX}$, $p22^{PHOX}$) and three cytosolic components ($p47^{PHOX}$, $p67^{PHOX}$, and $p40^{PHOX}$) in addition to the ancillary small GTPase Rac1. To test the requirement for NOX activity in rod induction by gp120, we employed a combination of pharmacological, molecular, and genetic approaches to block NOX activity. First, we used the NOX inhibitor TG6-227 (kindly provide by Dr. David Lambeth, Emory University GA) and exposed cells to dual-tropic gp120. We found rod formation was significantly reduced in TG6-227-treated neurons when compared to neurons exposed to gp120 in the absence of

inhibitor (p=0.002)(Figure 4A). Next, we infected mouse hippocampal neurons with adenovirus expressing the dominant-negative mutant of the NOX small membrane subunit $p22^{PHOX}$ ($DNp22^{PHOX}$) at 30 and 100 MOI (multiplicity of infection), thus inhibiting NOX activation. $DNp22^{PHOX}$-expressing hippocampal neurons showed no increase in rod induction when exposed to 500 pM dual-tropic gp120 at either MOI tested (Figure 4B). In contrast, both control neurons and those infected with adenovirus expressing the lacZ reporter (control infected) responded to dual-tropic gp120 with a nearly 4-fold increase in rods (Figure 4B). Lastly, we showed that the absence of the cytosolic membrane subunit $p47^{PHOX}$ ($p47^{PHOX}$-null mouse line) prevented rod formation induced by each gp120 strain (Figure 4C). Together these results demonstrate the inhibition of NOX activity is sufficient to block gp120-induced rod formation.

2.5. Discussion

Here we describe for the first time HIV gp120-mediated induction of cofilin-actin rods as a novel mechanism underlying synaptic dysfunction in HAND. Mouse hippocampal neurons were found to significantly increase both the percentage of neurons with rods as well as the number of rods in neurites following gp120 exposure. Rod induction requires gp120 interaction with R5 or X4 chemokine receptor and occurring through a pathway dependent on both PrP^C and NOX.

Cofilin:actin rod induction is a well-documented cellular response to neurodegenerative stimuli including mitochondrial dysfunction, ischemia/reperfusion, NMDA receptor mediated excitotoxicity, and the PrPc/NOX-dependent stimuli $A\beta_{d/t}$, pro-inflammatory cytokines, and now, gp120 (Chen et al., 2012; Walsh et al., 2014; Won et al., 2018).

Common to all rod inducers is the increased production of ROS in neurons exposed to these stimuli. We provide evidence that for gp120, activation of NOX which produces ROS is causal to rod formation supported by the absence of rod induction in cultures exposed to gp120 after preventing NOX2 activity through pharmacological inhibition, the introduction of dominant-negative mutation in the $p22^{PHOX}$ membrane subunit, or the gene-knockout of cytosolic subunit $p47^{PHOX}$. Furthermore, we demonstrate that PrP^C-null mouse hippocampal neurons do not generate rods in response to gp120 exposure. However, re-expressing PrP^C successfully recovered the potency of gp120 to induce rods in these cells. Notably, the neurotoxic $A\beta_{d/t}$ also requires NOX and is further dependent on the expression of PrP^C for induction of rods (Walsh et al., 2014).Taken together, these results provide evidence that rod induction via the PrP^C/NOX-dependent pathway may be a common mechanism underlying synaptic loss observed for a number of neurodegenerative diseases, including Alzheimer's disease and HAND.

HIV infection in the CNS appears restricted to microglia and macrophages while causing injury and apoptotic death in neurons (Jordan et al., 1991; Bell, 1998; Gerngross and Fischer, 2015). As such,

there has been some debate as to the mechanisms of neurotoxicity— whether neuronal injury observed in HAND is the result of indirect effects mediated by the release of neurotoxic, proinflammatory host factors from infected or activated glial cells, or rather the result of a direct neurotoxic effect of soluble HIV proteins shed from infected host cells and virus. Gp120 is a potent HIV neurotoxin implicated in promoting neurodegeneration through a combination of both indirect and direct actions (Kaul and Lipton, 1999; Wallace, 2006; Mocchetti et al., 2012; Smith et al., 2018). Gp120 has been shown to trigger the release of pro-inflammatory and pro-apoptotic cytokines, including TNF□, from infected macrophages and activated microglia, in an indirect mechanism of neuroinflammation (Guo et al., 2013; Festa et al., 2015; Planès et al., 2018). Mechanisms of direct neurotoxicity are mediated by gp120 interactions with host receptors at the neuronal membrane. In addition to its R5 and X4 co-receptors, gp120 has also been demonstrated to directly interact with neuronal NMDAR and □7-nAChR leading to increased intracellular calcium and induction of apoptosis (Bracci et al., 1992; Corasaniti et al., 2017).

Dissociated mouse hippocampal cultures are virtually devoid of microglia cells (Supplemental Figure S2) yet contain a population of astrocytes, which do not secrete detectable levels of proinflammatory cytokines as shown (Walsh et al., 2014). This allowed us to determine if gp120 interaction with chemokine co-receptors on the neuronal membrane activated the PrPc/NOX-mediated pathway of rod induction via a direct mechanism. Our results support gp120-mediated rod formation results from the direct interaction of gp120 and neuronal R5 or X4 receptors. Interestingly, while signaling through either R5 or X4 induced a significant increase in rod index, there appear to be differences in the strength of induction associated with individual receptors. Blocking either receptor from interacting with gp120 led to a reduction in rods induced by receptor-tropic gp120 strains tested; however, only AMD3100 inhibition of the X4 receptor significantly reduced rod induction to levels of untreated controls for both dual-tropic and X4-tropic strains of gp120. We did not observe a significant decline in rod production for cultures treated with maraviroc to block rod formation by dual-tropic or R5-tropic gp120. Indeed, it appears that blocking gp120/R5-mediated rod induction achieves only a partial block of rod induction. Though maraviroc treatment did achieve a reduction in rods, widespread cytotoxicity was observed in neuronal cultures treated with an initial concentration of 20 mM for inhibition of R5. To avoid neuron loss, the concentration of maraviroc was reduced to 100 nM, a concentration used widely in the literature. At 100 nM, we observed a small reduction in gp120 induced rod formation without apparent cytotoxicity. However, it is possible that at this lower maraviroc concentration we are not fully inhibiting R5 receptors, the remainder of which can then activate rod induction in response to gp120 binding. Notably, inhibiting R5 with maraviroc had no effect on rod formation activated via X4-mediated signaling. Likewise, X4 inhibition with AMD3100 had no effect on R5-mediated rod induction. Thus, rod

induction activated by different tropic-strains of gp120 is indeed receptor specific and independently activated by each receptor.

HAND in the post-cART era is no longer characterized by neuronal loss and brain atrophy; rather, neuronal synaptopathy has emerged as a hallmark of HIV-mediated neurotoxicity (Wenzel et al., 2019a). There is increasing evidence to support that impairments in neuronal cytoskeleton structure and function underlie synaptopathy observed in HAND and that it is the action of neurotoxic HIV proteins driving these impairments (Avdoshina et al., 2017; Avdoshina et al., 2019; Wenzel et al., 2019b). Though gp120 has been demonstrated to play a role in synaptic dysfunction and dendritic simplification thought to underlie cognitive impairments observed in HAND, the mechanisms underlying gp120-mediated synaptic damage are not well understood (Masliah et al., 1997; Iskander et al., 2004). The activation of a PrPc/NOX-mediated pathway of rod induction is one potential mechanism for gp120-induced synaptic dysfunction involving the perturbation of neuronal cytoskeleton dynamics.

Persistent rods have been observed during the progression of Alzheimer's and other neurodegenerative diseases (Minamide et al., 2000b; Chen et al., 2012; Rahman et al., 2014). In these pathologies, rods have been demonstrated to induce synapse loss by the interruption of vesicular transport due to occluded neurites (Maloney et al., 2005; Cichon et al., 2012). Rods have further been linked to synaptic dysfunction via sequestration of cofilin from dendritic spines where it has a role in post-synaptic plasticity (Gu et al., 2010; Rust, 2015)Thus, synaptopathy observed in HAND may arise from cytoskeletal aberrations induced by gp120 signaling through R5 and X4 in the form of cofilin-actin rods. Interestingly, a recent study reported dendritic simplification and cognitive flexibility in a transgenic rat model of HIV-1 infection can be rescued with the intracebroventricular administration of chemokine CXCL12, the endogenous ligand for the X4 receptor (Festa et al., 2020). Presumably, CXCL12 outcompetes gp120 to bind CXCR4, not only preventing gp120-associated synaptic toxicity, but also appearing to activate a pathway that seemingly reverses dendritic damage observed in HAND. That two different ligands signaling through X4 to generate opposite outcomes is not unheard of. Indeed, CXCL12 and gp120 signaling through X4 has previously been reported to differentially activate the spontaneous activity of Cajal-Retzius cells to opposite outcomes (Marchionni et al., 2012). In this study, the authors propose the ability of these CXCL12 and gp120 to modulate excitability of these cells in opposite directions results from the activation of distinct pools of intracellular calcium by each ligand. In the case of gp120/CXCL12 signaling and dendritic spine morphology, it may be that gp120 and CXCL12 signaling through X4 similarly activate different intracellular targets to modulate cytoskeletal effects.

These results provide evidence to support potential therapeutics that targeting rod formation could ameliorate not only HAND but also pathologic rod formation in response to several neurodegenerative stimuli, including Aβ oligomers and proinflammatory cytokines. Our results demonstrate gp120 co-

receptor antagonists— specifically AMD3100 inhibition of X4, would be a potential mechanism to block rod formation. Further, Festa et al. (2020) demonstrated CXCL12-mediated rescue of dendritic spines suggesting gp120 co-receptor agonists should also be considered for their potential to activate protective pathways against gp120-mediated toxicity. The importance of membrane architecture as a regulator of protein function in the gp120-mediated pathway to rod formation cannot be understated. Membrane lipid raft domains serve not only as critical sites of interaction for chemokine receptors, but are also essential for the appropriate receptor conformation to support co-receptor function (Nguyen and Taub, 2002; Jana and Pahan, 2004). Recent studies have attributed a 'chaperone-like' allosteric function for cholesterol in stabilizing structural elements of integral membrane proteins through a cholesterol recognition/interaction amino acid consensus sequence, with demonstrable alterations of protein function by modulating cholesterol interactions (Fantini, 2003; Fantini and Barrantes, 2013). Notably, putative cholesterol binding sites have been identified in the structure of both R5 and X4, supporting the necessity of lipid raft localization for proper receptor function (Zhukovsky et al., 2013; Legler et al., 2017). Further, both PrP^C and NOX2 are localized to raft domains where raft composition similarly affects conformational stability and enzyme activity (Taylor and Hooper, 2006; Jin et al., 2011; Botto et al., 2014). Lipid rafts have been implicated in the pathology of several neurodegenerative disorders, including Parkinson's, Huntington's, and Alzheimer's disease where altered raft composition appears to underlie changes in signal transduction contributing to pathogenesis (Sonnino et al., 2014; Grassi et al., 2019). As gp120-induced rod formation requires signaling not only through lipid-raft localized receptors, but also the involvement of proteins tied to raft domains, membrane architecture becomes an important potential target for drug discovery and therapeutics directed against HAND.

2.6. Figures and Tables

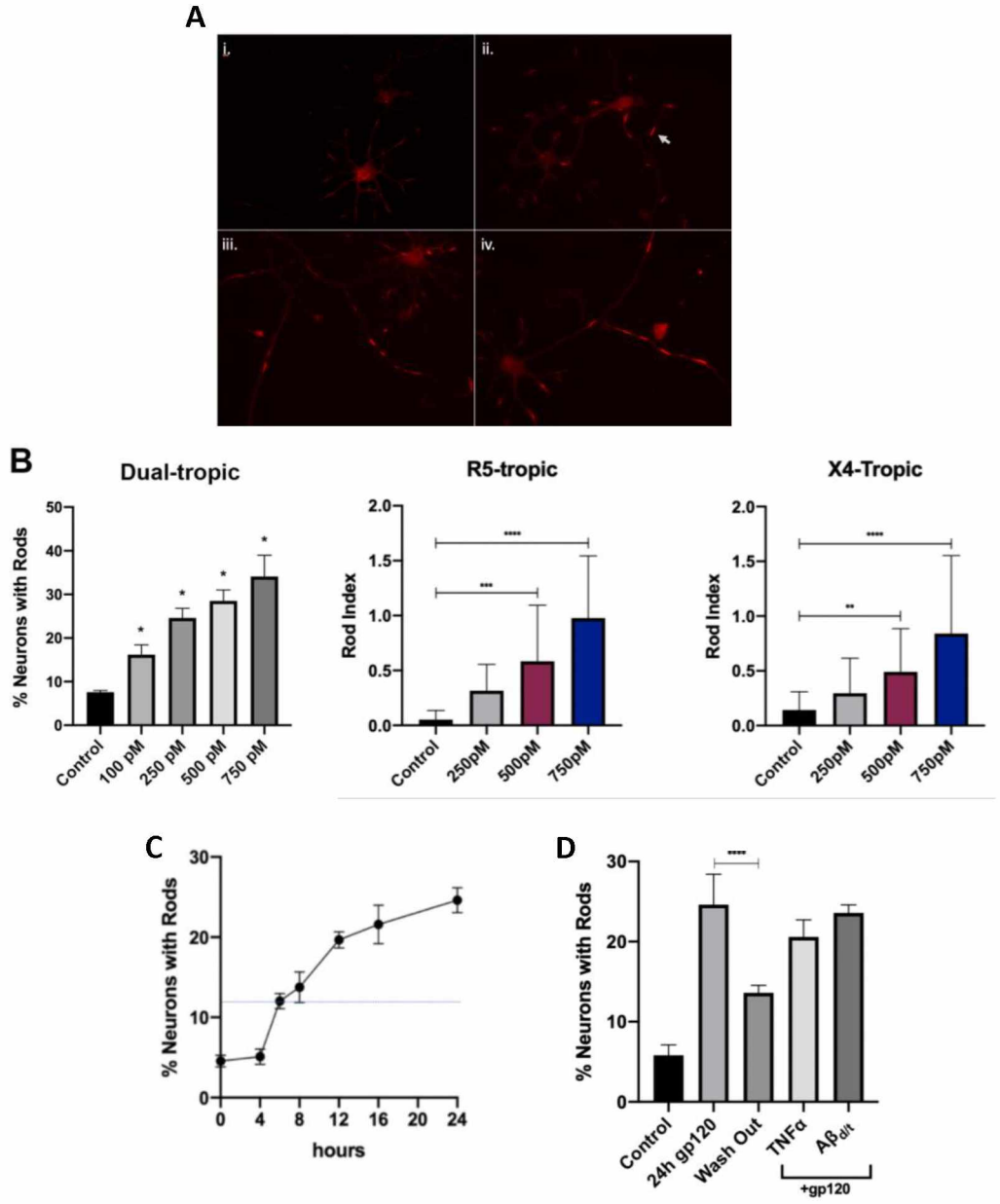


Figure 2.1. Dual-, R5-, and X4- tropic gp120 induce dose- and time-dependent rod formation in E16 mouse hippocampal neurons. A) Cells were grown on coverslips and treated with 250 pM dual-tropic gp120$_{MN}$ (ii), R5-tropic gp120$_{CM}$ (iii), X4-tropic gp120$_{IIIB}$ (iv) or untreated (i) for 16 hours then immunostained for cofilin and imaged. Each of the gp120 strains tested induced robust rod formation (*arrow indicates rod)*. B) Hippocampal neurons were treated with increasing concentrations of dual-, R5-, and X4- tropic strains of gp120 and processed as described above. Rods were found to form in a dose-dependent manner (* p<.01, ** p=.03, *** p=.0002, **** p<.0001). C) Percent of neurons expressing rods was quantified at 4, 6, 8, 12, 16, and 24 hours post exposure to 500 pM dual-tropic gp120. Gp120

induced rods significant above control from 6 hours onward (significance above dotted line p£.01). **D)** A wash-out of gp120 after 24 h significantly reduced rod formation (p<.0001). No additive effect was observed upon incubation with both gp120 and TNF-**α** or amyloid-β. *w-o = washout, GP+TNF = 500 pM gp120 & 50 ng/mL TNF-**α**, GP+Aβd/t= 500 pM gp120 & 200 pM Aβd/t*

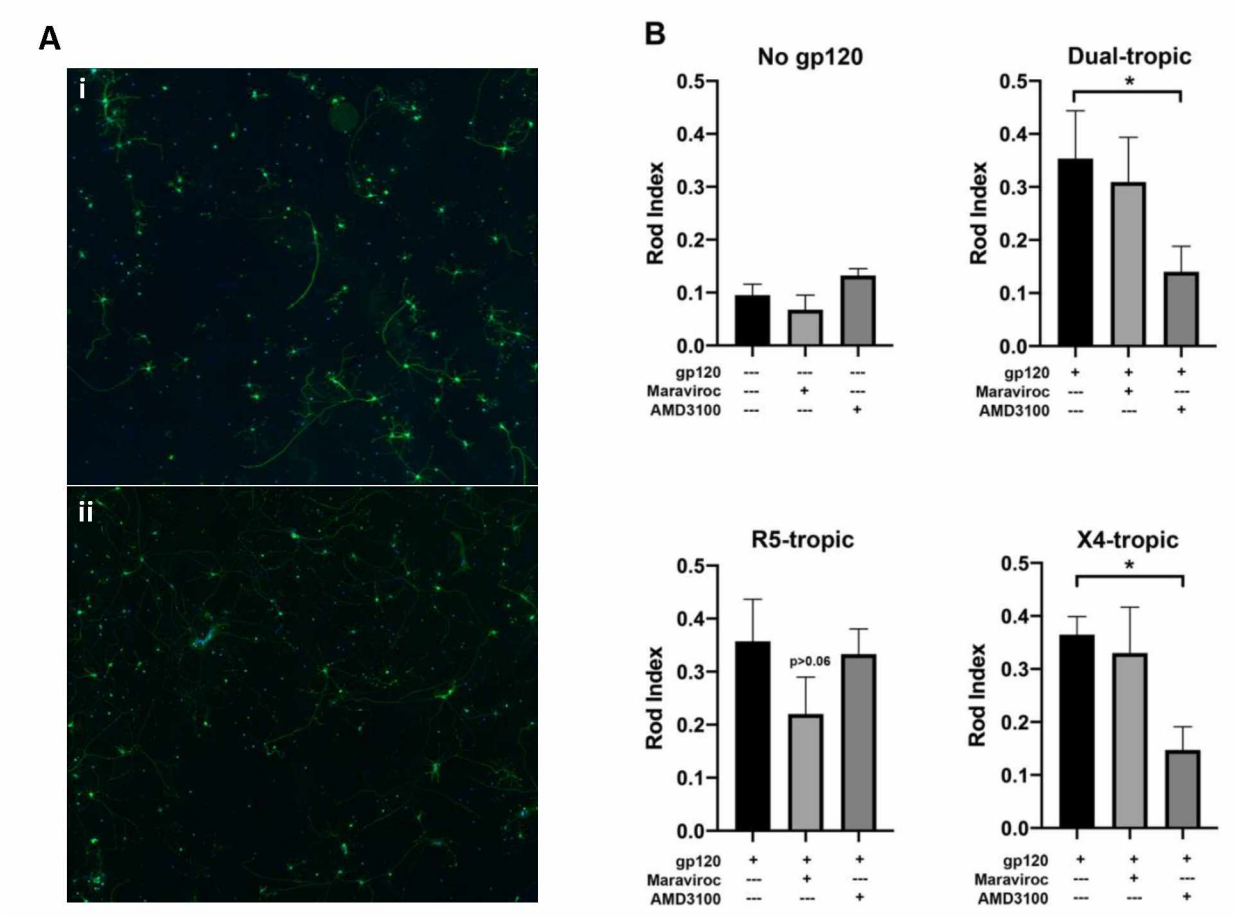


Figure 2.2. The CXCR4 receptor inhibitor AMD3100, but not CCR5 inhibitor maraviroc, blocks rod induction by CXCR4 binding gp120 strains. A) E16 mouse hippocampal neurons grown on coverslips were fixed on DIV7 and immunostained for CCR5 (i) and CXCR4 (ii). Both receptors were found to be widely expressed in these neurons. B) Neurons were pre-treated with 100 nM maraviroc or 50 nM AMD3100 for 1h prior to gp120 exposure and maintained throughout the 16-hour treatment. CXCR4 inhibitor blocked rod induction by X4-binding strains of gp120 returning the rod index to control levels ($p<.01$). CCR5 inhibitor maraviroc induced a nominal reduction in rod index of cells treated with R5-binding gp120 strains ($p=.06$).

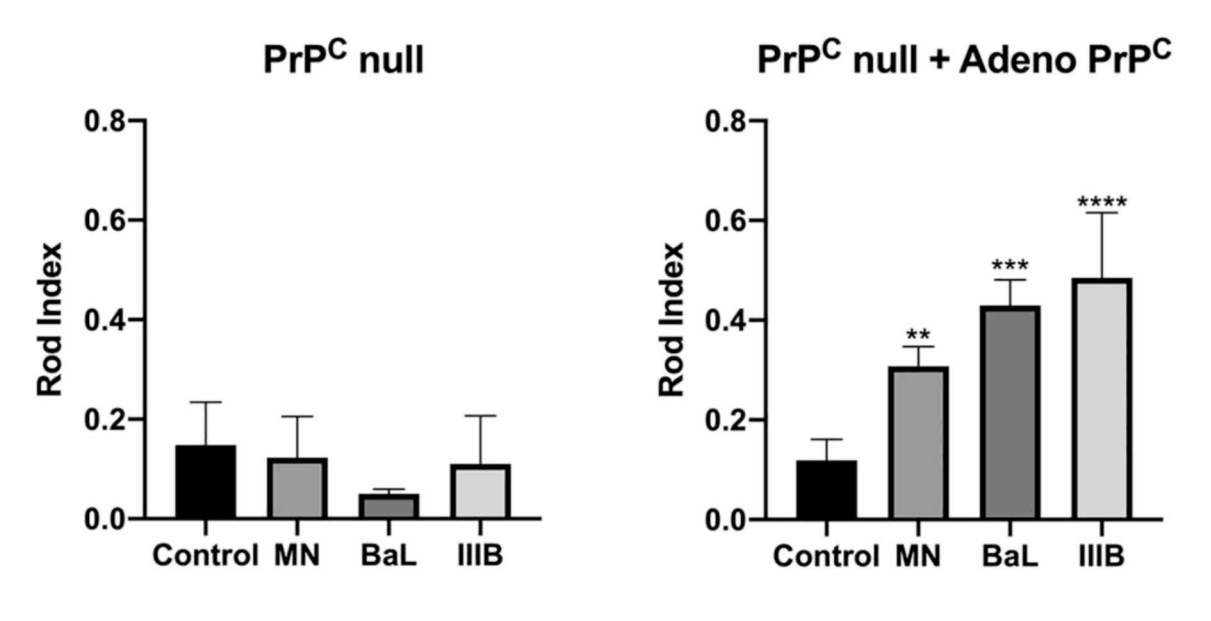


Figure 2.3. Gp120 mediated rod inductions requires the expression of cellular prion protein (PrPc). E16 mouse hippocampal neurons cultured from a PrPc-null mouse were exposed to each of the gp120 strains (250 pM, 16 h) and rod index calculated. None of the gp120 strains tested induced rod formation in PrPc-null neurons above control. PrPc-null neurons were then infected with adenovirus expressing EGFP-PrPc to drive re-expression of the protein. Infected cells were then exposed to 250 pM gp120 as described above and rod index calculated. In infected cells re-expressing PrPc, all strains of gp120 tested induced rod formation significant above control (** p=.0053, *** p=.0003, **** p<.0001).

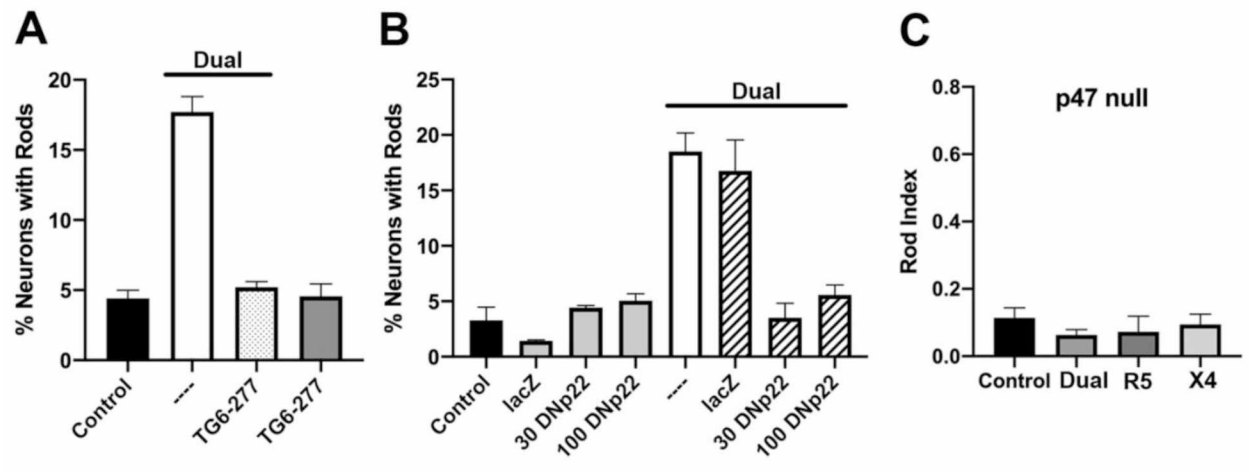
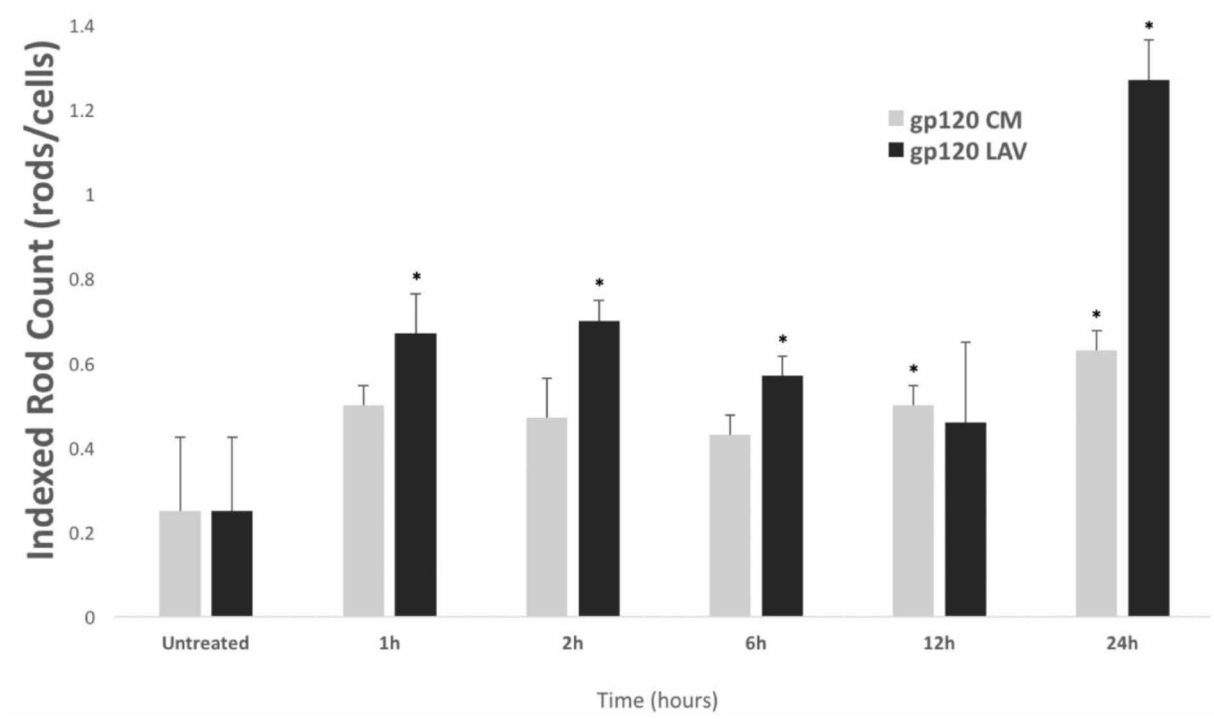
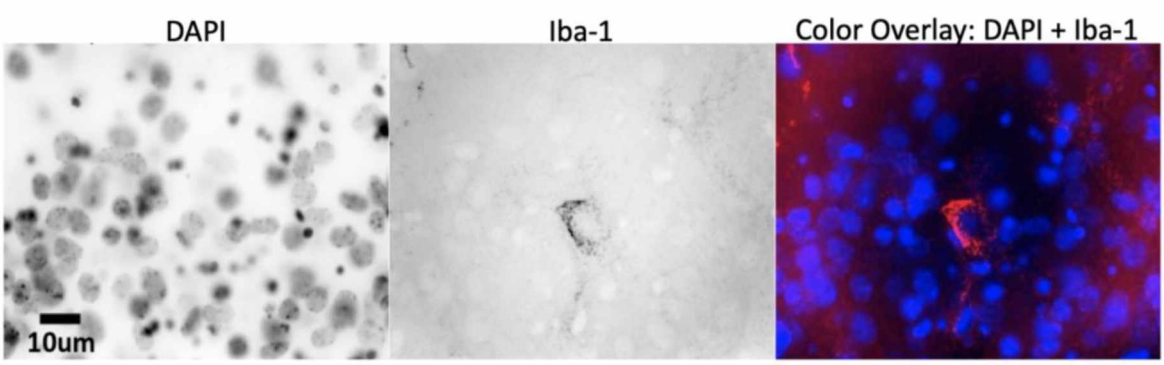


Figure 2.4. Gp120 induced rod formation occurs via a NOX2 mediated pathway. NOX2 activity was inhibited using pharmacologic, molecular and genetic approaches. A) The NOX2 specific inhibitor TG6-277 blocks dual-tropic gp120 mediated rod formation. B) Adenoviral infection of mouse hippocampal neurons with virus expressing dominant-negative mutant of NOX2 membrane subunit p22PHOX blocked dual-tropic gp120 induced rod formation at both MOIs tested. C) Hippocampal neurons from a cytosolic subunit p47PHOX knock-out mouse were exposed to gp120 and evaluated for rod formation. No strains of gp120 tested induced rod formation above control levels in p47PHOX-null neurons.



Supplemental Figure 2.1. R5- and X4-tropic gp120 induce time-dependent rod formation in E18 rat cortical neurons. Cells were grown on coverslips and treated with 250 pM R5-tropic gp120$_{CM}$ and X4-tropic gp120$_{IIIB}$ for indicated time then fixed, immunostained for cofilin, and imaged. While each of the gp120 strains tested induced rod formation, X4-tropic gp120 induced a more potent response, reaching significance above control at 1 hour post-exposure (* $p<.01$).



Supplemental Figure 2.2. Dissociated mouse hippocampal neurons are virtually devoid of microglia. E16.5 mouse hippocampal neurons were grown on coverslips, fixed on DIV7 and stained for DAPI which will stain nuclei of all cell types and Iba-1, a specific marker of microglia cells. An overlay of DAPI and Iba-1 reveals very few Iba-1 positive microglia in the culture.

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Chapter 3. Lipid Raft Coalescence Links Gp-120 Induced Oxidative Stress to Neurodegeneration

3.1. Abstract

HIV-associated neurocognitive disorders (HAND) affect nearly 50% of infected individuals. Current *in vitro* models for investigating the cellular and molecular mechanisms underlying HAND rely almost exclusively on the use of murine primary neurons and human neuronal cell lines. Despite the usefulness of these models, cells sourced from non-human hosts limit the applicability of results obtained, while human neuronal cell lines often exhibit neuropotential but not neuronal morphology. Notably, the SH-SY5Y human neuroblastoma cell line can be terminally differentiated into a phenotype expressing markers of mature neurons, as well as a morphology characteristic of mature neurons We sought to establish a human model of HIV envelope protein gp120-induced neurotoxicity by using human SH-SY5Y neuroblastoma cells to investigate the ability of gp120 to activate a pathway demonstrated to lead to the formation of pathologic cofilin-actin rods in primary murine neurons. To this end, we assessed cellular response to gp120 exposure by measuring lipid raft coalescence and the generation of reactive oxygen species, and further sought to assess the applicability of terminally differentiated SH-SY5Y cells as a reproducible neuronal model of human origin for the investigation of gp120 induced rod formation. We demonstrate here that gp120 induced lipid-raft coalescence in SH-SY5Y cells occurs in a dose-dependent manner. Moreover, gp120 exposure activated NOX2 and a concomitant generation of superoxide. While these are critical steps in the activation of rod induction in murine neurons, we did not observe any effect of gp120 on the production of cofilin-actin rods in differentiated SH-SY5Y cells.

3.2. Introduction

Nearly 50% of individuals with long-term HIV infection are affected by the onset of progressive neurological and cognitive complications referred to as HIV-associated neurocognitive disorders (HAND). While improvements in HIV treatments have contributed to a decline in the incidence of the severe dementias previously associated with HAND, the prevalence of milder forms of HAND remain stable and continue to have profound impacts on the quality of life for infected individuals. Indeed, the prevalence of HAND is expected to rise with the ageing population of HIV infected individuals— in the United States alone, of the 1.2 million people living with HIV infection, an estimated 50% are older than 50 years of age (Wing, 2016; Negredo et al., 2017). It has become apparent that complications from HIV infection

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overlap with other age-associated neurodegenerative diseases, including Alzheimer's disease, with evidence increasingly indicating that HIV-1 infection contributes to age-associated cognitive decline (Green et al., 2005; Achim et al., 2009; Cohen et al., 2015; McLaurin et al., 2019). As such, complications of long-term HIV infection, including HAND, remain a continuing public health challenge in the post-cART era (Alford and Vera, 2018).

HIV infiltrates the CNS early during primary infection where it establishes persistent infection in resident macrophages and glial cells that in turn release inflammatory cytokines and neurotoxic viral proteins that can perturb neuronal function (Gras and Kaul, 2010). The viral envelope protein gp120 has been identified as a potent neurotoxin affecting neurodegeneration not only via indirect mechanisms, but also through the direct interaction of the protein with host-cell chemokine co-receptors CCR5 and CXCR4 where preferential binding of gp120 to CCR5 or CXCR4 determines viral tropism (Smith et al., 2018). The interaction of gp120 with co-receptors has been demonstrated to directly affect neurodegeneration through multiple pathways, including activation of neuronal apoptosis via interaction with N-methyl-d-aspartate receptors (NMDAR), the induction of neuronal reactive oxygen species (ROS) and nitrosative stress, and axonal degeneration mediated by co-receptor activation. (Pattarini et al., 1998; Kaul et al., 2001; Jana and Pahan, 2004; Melli et al., 2006; Pandhare et al., 2015). While synaptic disruption is frequently observed in conjunction with apoptosis, in the absence of neuronal loss synaptodendritic injury is likely to be underlying the cognitive decline seen in HAND (Ru and Tang, 2017; Guha et al., 2018; Mackiewicz et al., 2019).

Notably, we have identified a pathway leading to synaptic and dendritic dysfunction mediated by gp120 interaction with CCR5 and CXCR4 co-receptors in rodent hippocampal and cortical neurons (Chapter 2). This pathway appears common to Alzheimer's associated amyloid-β (Aβ) oligomers and proinflammatory cytokines and is composed of the cellular prion protein (PrP^C) and NADPH oxidase-2 (NOX2) embedded in coalesced lipid raft macrodomains (Walsh et al., 2014). NOX2 is comprised of 6 subunits— 2 integral membrane proteins, 3 cytosolic regulatory proteins, and a guanine nucleotide binding protein— that interact to form the active enzyme complex (Panday et al., 2015). Upon activation, cytosolic regulatory proteins, including $p67^{PHOX}$, translocate to the plasma membrane and complex integral membrane proteins to assembly the active enzyme. Subsequent ROS generation is causative to the formation and accumulation of rod-like cofilin-actin inclusions (rods) in neuronal processes, which have been linked to synaptic dysfunction via sequestration of cofilin as well as disruption of vesicular transport resulting from the occlusion of neurites containing rods (Bamburg et al., 2010).

Murine cells are commonly used for the investigation of both the cellular and molecular mechanisms underlying both HAND and pathologic rod formation in response to various stimuli. These cells are a useful alternative to primary human neurons owing to the expense and ethical issues involved

in obtaining primary neuronal cells of human origin. However, these non-human origins may affect experimental outcomes and limit the applicability of results, particularly for viruses specific to human hosts, like HIV. Indeed, we have identified differences in chemokine receptor expression between rodent and human neurons that have the potential to confound results obtained from experiments requiring direct gp120-receptor interactions. To overcome the limitations associated with the use of animal cells in the investigation of a human disease, we proposed to evaluate the ability of gp120 to induce cofilin-actin rods in neurons of human origin.

The human neuroblastoma cell line SH-SY5Y has been widely used to model human neurodegenerative disease, including HAND. While in their undifferentiated form SH-SY5Y cells rapidly proliferate and exhibit short, spiky processes, these cells can be differentiated to exhibit long, branched processes more typical of primary neurons (Shipley et al., 2016). This is of particular importance when investigating gp120 mediated rod formation, as pathologic rod formation occurs in neuritic processes, and thus any cellular model for rod formation requires the long processes seen in primary neurons. Here, we present data to show that consistent with data obtained in murine primary neurons, undifferentiated SH-SY5Y cells responded to gp120 exposure with a marked coalescence of lipid raft into macrodomains and the rapid generation of NOX2-mediated ROS production. Further, we evaluate differentiated SH-SY5Y cells as a model for gp120 induced rod formation in cells of human neurons.

3.3. Materials and Methods

3.3.1. Chemicals, Biological, Antibodies

R5-tropic gp120$_{CM}$ and X4-tropic gp120$_{LAV}$ were purchased from ProSpecBio (Ness-Ziona, Israel) and dual-tropic gp120$_{MN}$ purchased from ImmunoDX (Woburn, MA). Vybrant™ AlexaFluor™ 594 Lipid Raft labeling kit and Cholera toxin subunit B (recombinant) Alexa Fluor™ 488 conjugate were purchased from ThermoFisher Scientific (Waltham, MA). Primary anti-p67 phox antibody was purchased from Abcam (Cambridge, MA). Recombinant human TNFa was received from ProSpecBio (Ness-Ziona, Israel). 2',7dihydrodicholorfluorecein diacetate (H_2DCFDA), Dihydroethidium (DHE), and PrestoBlue Cell Viability reagent were purchased from ThermoFisher Scientific (Waltham, MA).

3.3.2. Cell culture

SH-SY5Y cells were obtained from American Type Culture Collection (ATCC CRL-226) and maintained in the undifferentiated phenotype using EMEM with 2mM L-glutamine (ThermoFisher) supplemented with 10% Hyclone fetal bovine serum (VWR) and 100 U/mL penicillin/streptomycin (VWR) at 37$_{o}$C as a growth medium. *Differentiating SH-SY5Y Cells.* To differentiate SH-SY5Y cells into a more neuronal phenotype, a previously described protocol was employed (Shipley et al., 2016). Briefly,

SH-SY5Y cells were grown on extracellular matrix (MaxGel ECM, Sigma, St. Louis, MO)- coated 12 mm glass coverslips subjected to three different differentiation mediums containing sequentially less serum and including 10mM retinoic acid. The protocol was modified to reduce incubation duration in each differentiation medium, reducing time periods from 6 days to 4 days and decreasing overall time to differentiation from 18 days to 12 days (see figure 3.3. for greater detail).

3.3.3. Lipid raft assays

For imaging lipid rafts, SH-SY5Y cells were grown on rat collagen coated coverslips until 80-90% confluent. Cells were serum starved for 24 hours prior to treatment with gp120. Cells were exposed to gp120 by complete media exchange and gp120 was maintained in culture medium for the duration of experiments. Lipid rafts were visualized using Vybrant AlexaFluor 594 Lipid Raft labeling kit according to manufacturer's directions, stained for DAPI, and fixed in 4% paraformaldehyde. Images were acquired using an Olympus Inverted Research Microscope (motorized Model 1X81) using SlideBook Software and analyzed using MetaMorph software. To quantify raft coalescence, images were converted to grayscale (8 bit), binarized, and overlaid with the original image to isolate lipid raft domains in regions of interest. The mean integrated intensity of all rafts per condition was calculated and used to determine significance. Lipid raft coalescence was also quantified using a plate reader approach. SH-SY5Y cells were grown in black 96-well plates with clear bottoms and exposed to 500 pM of each gp120 strain for 2.5, 5, and 24 hours. Lipid rafts were labelled with Alexa488 conjugated cholera toxin subunit B and fluorescence detected using a multiplate reader (Beckman Coulter Multimode DTX 880 microplate reader).

3.3.4. ROS assays

Formation of intracellular ROS were quantified using the oxidation sensitive fluorescence indicators H_2DCF-DA and DHE. H_2DCF-DA detects the general presence of ROS, while DHE provides a superoxide (O_2-) specific measure of ROS. With regard to H_2DCF-DA, SH-SY5Y cells grown in 96 well plates were loaded with 1 μM H_2DCF-DA in serum-free DMEM (1 h, 37°C) followed by exposure to 250 pM and 500 pM of either R5-tropic or X4-tropic gp120. DCF fluorescence was measured starting at 5 min post addition and every 15 min thereafter, for a total of 155 minutes, by plate reader (Beckman Coulter Multimode DTX 880 microplate reader). Total DCF fluorescence (ex/em 495/525) from each well was adjusted to the number of viable cells using PrestoBlue (cell viability reagent, absorbance measured at 570 nm) and normalized to control. With regard to DHE, SH-SY5Y cells were loaded with 5□M H_2Et (100 μl total volume/well, 30 min, 37°C) in the dark due to the light sensitivity of DHE. Cells were rinsed once with serum free EMEM prior to exposure to 250, 500, and 1000 pM R5 or X4-tropic g120 for 2.5 h. Cells were rinsed 2X with warm HBSS and medium replaced with 100 μl Leibovitz

medium without phenol red. Excitation/emission was measured at 590/620 for the detection of O_2^- specific oxidation by plate reader (described above).

3.3.5. Biotinylation of membrane proteins and plasma membrane association of $p67^{PHOX}$

SH-SY5Y cells were grown to confluence in 6-well plates and treated with 250 pM dual-tropic gp120 for 10 or 30 min. Following gp120 exposures, plasma membrane proteins were biotinylated by the method described (Li and Shah, 2002). Briefly, cultures were rinsed and incubated on ice with 0.5mg/mL membrane impermeable EZ-Link™-NHS-SS-Biotin (ThermoFisher Scientific) in a HEPES buffer . Next, cells were scraped into solution, centrifuged (200xg_{max}, 2 min), and gently lysed (TBS w/ 0.5% TritonX) by sonicatation (on ice). Cells lysates were cleared by centrifugation (24,000xg_{max}, 5min, 4°C) and supernatants transferred to fresh microcentrifuge tubes. Each lysate (500 µl) was incubated with 20 µl streptavidin agarose beads (ThermoFisher Scientific, Waltham, MA) for 1 h at RT while rocking. Beads were pelleted (5,000g_{max}, 5min, 4°C) and washed twice with TBST (500 µl /wash). Bound protein was released from beads by resuspending in 400uL TBST with 50mM DTT (freshly made) and incubating for 1h at RT while rocking. After incubation, beads were spun at 5,000g_{max} for 5 min (4°C) and supernatants recovered and used to coat a 96-well ELISA plate (80uL protein suspension/well) overnight at 4°C. before incubation with primary antibody against p67phox (1 h, RT) then followed by HRP-conjugated secondary antibody (1:100, 1 h RT) . Plate was washed 3X before incubating with 1-Step™ Ultra TMB-ELISA Substrate Solution (ThermoFisher Scientific, Waltham, MA) for 15 minutes (or until blue coloration is visible) and reaction stopped with 1M H_2SO_4. Absorbance was measured at 450nm using a Beckman Coulter Multimode DTX 880 microplate reader. Data was adjusted to protein expression of NCAM used as an internal control.

3.3.6. Immunostaining for cofilin-actin rods

SH-SY5Y cells were grown on 12 mm round glass coverslips inserted into a 24 well plates and differentiated to D-8 and D-12. Cultures were treated with 250 pM dual-tropic gp120 (18 h) prior to fixation in 4%formaldahyde/0.1%glutaraldahyde for 20 minutes and permeabilization with ice-cold methanol for 3 minutes. Coverslips were rinsed with PBS (3X) and blocked in 1%BSA and goat serum in TBS (1 h). Coverslips were then incubated with a rabbit anti-cofilin antibody (1:100 concentration, AbCam, Cambridge, MA) overnight at 4°C. Coverslips were rinsed 3 x with PBS and incubated with Alexa488-conjugated anti-rabbit secondary antibody (1:500, 1 h, RT)), washed and mounted on glass slides using pro-long gold antifade cell mounting media with DAPI (ThermoFisher). Images were acquired using the Olympus Inverted Research Microscope (motorized Model 1X81) at 600X magnification.

3.3.7. Statistical analysis

Quantification of all experiments were performed with at least triplicate samples for each condition and repeated in at least three independent experiments with the exception of biotinylation and membrane localization of p67PHOX, which was repeated in two independent experiments. Lipid raft image data were assessed for Gaussian distribution and raft coalescence analyzed by Kruskal Wallis test with Dunn's post-hoc analysis for multiple comparisons (non-parametric data) using GraphPad Prism software. 96-well plate-based raft coalescence, DCFDA and DHE ROS production, and rod formation were analyzed by one-way ANOVA followed by Dunnett's post-hoc analysis with GraphPad Prism (San Diego, CA) software. An alpha value of 0.05 for used for all analyses.

3.4. Results

3.4.1. Gp120 induces dose-dependent lipid raft coalescence in SH-SY5Y neuroblastoma cells.

Gp120 interaction with host cell receptors is restricted to membrane lipid raft domains with rafts serving to compartmentalize cellular processes and facilitate protein-lipid / protein-protein interactions and signal transduction events. Raft coalescence has been proposed as a mechanism for clustering the components of receptor-activated signaling cascades, and gp120 has previously been demonstrated to induce raft coalescence in human hippocampal neurons (Jana and Pahan, 2004). Notably, PrP^C is localized to lipid rafts, where the early association of the protein with the cholesterol-enriched environment is thought to facilitate its proper folding (Sarnataro et al., 2004). Further, activation of NOX2— and in particular the assembly of its cytosolic subunits $p40^{PHOX}$, $p47^{PHOX}$, and $p67^{PHOX}$, together with Rac1 — has been shown in most cell types to be dependent on the lipid raft environment (Haslund-Vinding et al., 2017). Indeed, both HIV co-receptors CCR5 and CXCR4 have been demonstrated to localize to lipid rafts in response to gp120 exposure, where interaction with membrane cholesterol supports the proper conformation and activity of the receptors (Nguyen and Taub, 2002; Yi et al., 2006; Kamiyama et al., 2009). As both PrP^C expression and NOX2 activity are required for rod induction by gp120 in rodent neurons, we tested the hypothesis that gp120 binding to co-receptors CCR5 and CXCR4 would lead to an increased size and stability of raft domains. SH-SY5Y cells grown on glass coverslips were exposed to increasing doses of dual-tropic $gp120^{MN}$ for 2.5 hours and lipid rafts visualized with Alexa564-labeled Cholera toxin subunit B (CTXB), which binds the lipid-raft anchored ganglioside GM1. We found that raft coalescence in SH-SY5Y cells significantly increased in a dose-dependent manner when exposed to dual tropic $gp120^{MN}$ ($p<.0002$) (Figure 1A). The pro-inflammatory cytokine TNFα which has previously been demonstrated to induce lipid raft coalescence and cofilin-actin rod formation (Gustafson et al., 2012; Walsh et al., 2014), similarly induced the coalescence of lipid rafts in SH-SY5Y cells ($p<.0001$). Having demonstrated that gp120 is capable of inducing lipid raft coalescence

in these cells, we next explored the effect of each gp120 strain on lipid raft coalescence as a function of time. SH-SY5Y cells grown in 96-well plates were exposed to 500 pM dual-tropic $gp120_{MN}$, R5-tropic $gp120_{CM}$, and the X4-tropic $gp120_{LAV}$ for a duration of 2.5 h, 5h, and 24 h before fluorescently labeling lipid rafts with Alexa488-conjugated CTXB and quantifying lipid raft coalescence. We found that while all strains of gp120 tested induced significant lipid raft coalescence by 5 hours post-exposure ($p<.0001$), any measurable differences in raft size between exposed and unexposed cells were lost at 24 hours post-exposure (data not shown), suggesting that gp120 induced raft coalescence and any associated activation of signaling is not sustained long term (Figure 1B).

3.4.2. Gp120 activates NOX2 to generate ROS

ROS plays a critical role in the pathway to formation of cofilin-actin rods, where oxidation induced changes in cofilin lead to the formation of disulfide-linked cofilin dimers necessary for bundling cofilin-actin filaments into rods (Bamburg et al., 2010). To confirm that SH-SY5Y cells generate ROS in response to gp120 stimulation as observed in murine neurons, we exposed cells in 96-well plates to two concentrations of gp120 for both R5-tropic and X4-tropic strains and measured ROS production with the fluorescent ROS-indicator 2',7' –dichlorofluorescin diacetate (DCF-DA). Both strains of gp120 tested induced a temporary, rapid production of ROS in SH-SY5Y cells with levels returning to those of control by 2.5 hours post-exposure (Figure 3.2A). ROS production was greatest within five minutes of exposure ($p\leq0.05$) and notably, 500 pM of X4-tropic $gp120_{LAV}$ induced a 14% greater rise in ROS as compared to 500 pM R5-tropic $gp120_{CM}$. This is in accordance with previous results obtained in murine primary neurons that suggest gp120 signaling through CXCR4 is a more potent rod inducer (manuscript in preparation). As NOX2 generates ROS via a redox reaction with molecular oxygen and NADPH to produce superoxide (O_2^-), we next evaluated O_2^- production in SH-SY5Y cells using the superoxide-selective indicator dihydroethidium (DHE) at 2.5 h following exposure to increasing concentrations of dual-tropic $gp120_{MN}$. Our results revealed that while gp120 induced superoxide significantly above control at 2.5 hours post-exposure ($p\leq.004$)(Figure 2B). Having confirmed gp120 induced production of superoxide, we next sought to confirm activation of NOX2 by assessing increased plasma membrane association of cytosolic subunits. To this end, we exposed SH-SY5Y cells grown in 6-well plates to dual-tropic $gp120_{MN}$ for 10 and 30 minutes before biotinylating plasma membrane proteins. Membrane-associated proteins were collected following gentle cell lysis and a streptavidin-agarose bead pull down. Bound material was washed, released with DTT, and used to coat a 96-well ELISA plate to detect for the cytosolic NOX2 subunit $p67^{PHOX}$. We hypothesized gp120 activation of NOX2 would lead to a detectable increase of membrane-associated $p67^{PHOX}$ in exposed SH-SY5Y cells. We confirmed that gp120 is indeed activating NOX2, with a nominal increase of membrane-associated $p67^{PHOX}$ detectable by 10 minutes post

exposure (Figure 3.2C). Taken together, these results confirm that gp120 is activating NOX2-mediated production of superoxide in SH-SY5Y neuroblastoma cells, similar to what has been observed in the pathway to rod induction in primary rodent neurons.

3.4.3. Differentiated SH-SY5Y human neuroblastoma cells do not form rods in response to gp120 exposure

To determine if gp120 signaling through CCR5 and/or CXCR4 is inducing the formation of rods in neurons of human origin, we sought to test the ability of all three gp120 strains in generating rods in SH-SY5Y cells. While undifferentiated SH-SY5Y cells exhibit truncated, immature processes (Figure 3.3A), these cells can be differentiated to a more neuronal phenotype with long processes (Figure 3.3D) by a variety of methods, typically involving the gradual starvation of serum and the introduction of retinoic acid (RA) (Kovalevich and Langford, 2013). We chose to differentiate SH-SY5Y cells using a procedure adapted from Shipley et al, 2016 that involved the sequential removal of serum, continued presence of RA, and addition of neurotrophic factors including brain-derived neurotrophic factor (BDNF) from/to culture medium (Shipley et al., 2016). While the original procedure described an eighteen-day differentiation period with two re-plating steps, we reduced the number of days to differentiation to twelve, and plated cells directly onto extracellular matrix coated glass coverslips, eliminating the need for re-plating. We found these modifications improved the viability of our cultures without affecting differentiation (Figure 3.3). SH-SY5Y cells were plated on 12mm glass-coverslips coated in extracellular matrix and differentiated according to the modified procedure. Cells on coverslips were selected from two time points during differentiation— 8 days differentiated and 12 days differentiated, designated D-8 and D-12 (Figure 3.3C and 3.3D, respectively). For both conditions, cells were exposed to 250 pM dual-tropic, R5-tropic, and X4-tropic gp120 for 18 hours before being fixed and immunostained against cofilin and evaluated for rod formation. However, for all strains of gp120 at the concentration tested, we were unable to detect rod formation at either stage of differentiation evaluated (Figure 3.4).

3.5. Discussion

Here, we demonstrated gp120 signaling through both CCR5 and CXCR4 induced a dose-dependent increase in coalesced lipid rafts and NOX2-medaited generation of superoxide in a human neuron-like model system. Though we confirmed similarities in the pathway to rod induction previously described for rodent hippocampal and cortical neurons, we were unable to recapitulate rod pathology in differentiated SH-SY5Y cell line.

The use of animal models to explore cellular and molecular mechanisms underlying disease has contributed much to the understanding of disease pathogenesis and the development of new targets for

therapeutic interventions. While animal cells are useful models for elements of human disease, they are not reflective of the entire etiology of the disease. Indeed, there is a high failure rate for the translation of therapeutics developed in animal models to humans (Bracken, 2009). Most current in vitro models for investigating rod formation rely on the use of murine primary neurons, while neuronal models used in the investigation of HAND often include neuron-like cells that lack the long processes more characteristics of mature neurons. Notably, human SH-SY5Y neuroblastoma cells can be terminally differentiated into cells expressing markers of mature neurons with long, branching processes, thus proving an attractive potential model for rod pathology in HAND. That we did not observe rod formation in differentiated SH-SY5Y cells is not indicative of an inability for human neurons to form rods in response to inducing stimuli—rods have previously been described to form in the post-mortem brains of individuals with Alzheimer's disease (Minamide et al., 2000). Rather, there are a number of possible factors affecting rod formation in SH-SY5Y cells, including the possibility that given its origins as a cancer cell line, SH-SY5Y neuroblastoma cells are more robust to gp120 toxicity than primary murine neurons, thus requiring a greater concentration of gp120 than was tested to activate the pathway to rod formation. It is also possible that cell density affected the ability to detect rod formation. In cultures differentiated for the entire 12-day protocol exposed to gp120 (Figure 3.4D), neurons were so densely interconnected that a three-dimensional meshwork of neurite outgrowth formed, making the detection of potential rods very difficult. Future experiments should adjust plating density per 12mm glass coverslip from 10,000 cells/well to 5,000 cells/well to allow for more space between cell bodies for neurite outgrowth. Finally, it is possible that SH-SY5Y cells, though of human origin, do not fully recapitulate the biology of disease as a consequence of immortality, where repeated rounds of replication introduce mutations and genetic drift that can potentially affect neuronal responses to various stimuli. This is supported by a study demonstrating SH-SY5Y cells do not endogenously express the PrP^C protein. Notably, transfecting these SH-SY5Y to stably express PrP^C rescued PrP^C-mediated intracellular zinc transport (Watt et al., 2012). It is possible the absence of PrP^C prevented gp120-mediated rod formation in differentiated SH-SY5Y cells, as the pathway to rod induction in murine neurons has been shown to require the expression of PrP^C. Thus, differentiated SH-SY5Y cells as a model for gp120 induced rod pathology may still be viable with the re-expression of PrP^C, continued culture optimization, and dose-dependence studies with gp120 and other known inducers of rod formation.

The prevalence of neurological complications associated with prolonged HIV infection are expected to rise with the aging population of people living with HIV. Improving neurological outcomes in these individuals requires a better understanding of the molecular mechanisms underlying HIV-induced neurodegeneration and developing appropriate neuronal models for the investigation of the cellular mechanisms underlying HAND pathogenesis is a key step in improving our understanding of the disease.

Importantly, we demonstrate gp120 induces lipid raft coalescence in a dose-dependent manner in SH-SY5Y cells. Gp120 also initiated the rapid production of ROS in these cells, and levels of superoxide were found to be elevated above control 2.5 hours post gp120 exposure. The detection of superoxide suggested the involvement of NOX2 activity, which was confirmed by demonstrating gp120 triggers the translocation of the NOX cytosolic regulatory protein $p67^{PHOX}$, to the plasma membrane. These findings link gp120 signaling via lipid raft associated receptors with NOX2 generated superoxide, and potentially to the formation of cofilin-actin rods.

3.6. Figures and Tables

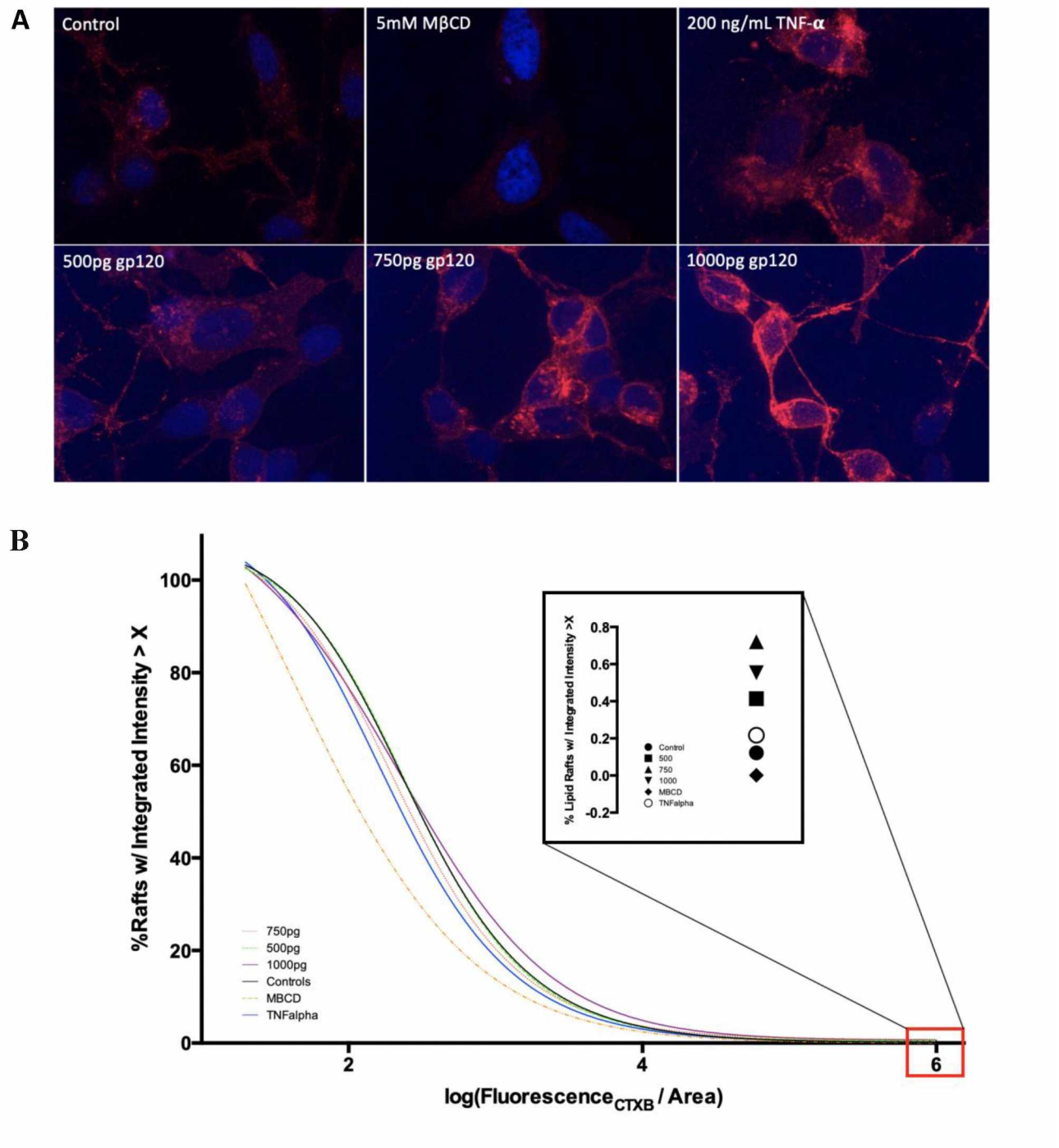

Figure 3.1. All strains of gp120 induce lipid raft coalescence in SH-SY5Y neuroblastoma cells. A. SH-SY5Y cells grown on glass coverslips were treated with 500 pM, 750 pM, and 1000 pM of dual-tropic gp120 for 2.5 hours.

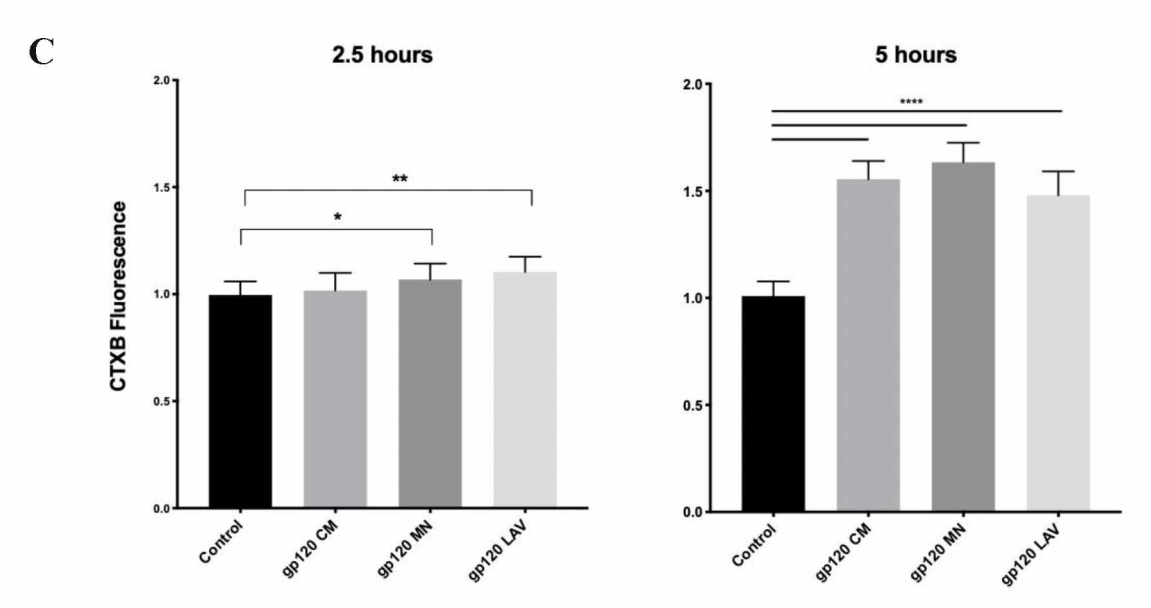

Figure 3.1 cont. Cells were also treated with 5 mM of the cholesterol chelator methyl-β-cyclodextrin, known to disrupt rafts, and 200 ng/mL TNFα, known to trigger lipid raft coalescence, or were left untreated as control. B. Representative curves of integrated intensity ($Fluorescence_{CTXB}$/Area) values for gp120 treatments at 2.5 hours. Integrated intensity of the raft-stained SH-SY5Y cells was determined using MetaMorph image analysis software. Images were converted to grayscale and used to create binary masks to define threshold lipid raft domains in regions of interest. Overall cumulative frequency distribution is shown for each condition as the percentage of lipid rafts expressing integrated intensity values greater than X over the log of integrated intensity. Magnified portion shows the comparison of percent frequency of lipid rafts at X=6. Exposing SH-SY5Y cells to 750 and 1000pg of dual-tropic $gp120_{MN}$ induced a significant increase in lipid raft size (Kruskal-Wallis, $p<.0002$). C. SH-SY5Y neurons were grown in 96-well plates and exposed to 500 pM of R5-tropic $gp120_{CM}$, dual-tropic $gp120_{MN}$, and X4-tropic $gp120_{LAV}$ for 2.5, 5, and 24 hours. All strains tested induced significant increase in raft coalescence above control by 5-hours post exposure ($p<.0001$). This effect was lost by 24-hours post exposure (data not shown).

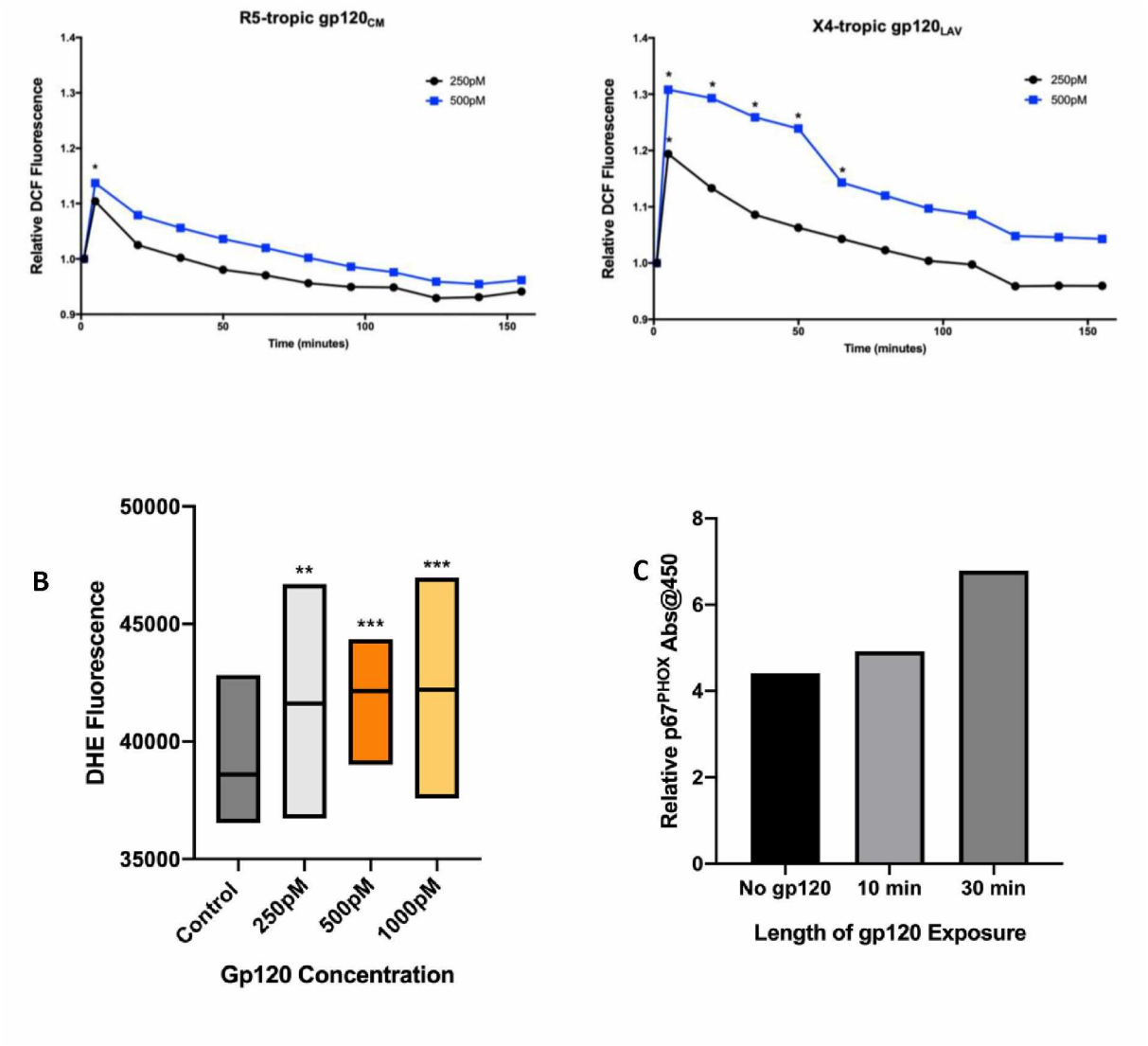

Figure 3.2. Gp120 activates NOX2 and a concomitant formation of superoxide. A. SH-SY5Y cells grown in 96 well plates were exposed to 250 and 500 pM concentrations of R5-tropic or X4-tropic gp120 and maximum DCF fluorescence/well was measured 5 min post addition and every fifteen minutes thereafter, for a total of 155 minutes. ROS production was greatest immediately following treatment (*p≤0.05). B. SH-SY5Y cells in 96 well plates were treated with increasing concentrations (250, 500, 1000 pM) of dual-tropic gp120 for 2.5 h and production of superoxide detected using dihydroethidium. Superoxide levels were significant above control for all concentrations of gp120 tested (**p=.004, ***p=.0005). C. Cells were grown in 6-well plates and treated with dual-tropic

gp120 for 10 and 30 minutes before biotinylating plasma membrane proteins. After cell lysis, biotinylated proteins were collected via a streptavidin-agarose bead pull down. Bound protein was released and used to coat a 96-well ELISA plate and was then assayed for the cytosolic NOX2 subunit p67PHOX. A nominal increase in translocation of the cytosolic subunit p67PHOX to the plasma membrane at both 10 and 30 minutes post-exposure to gp120 was observed, though it did not reach the level of statistical significance.

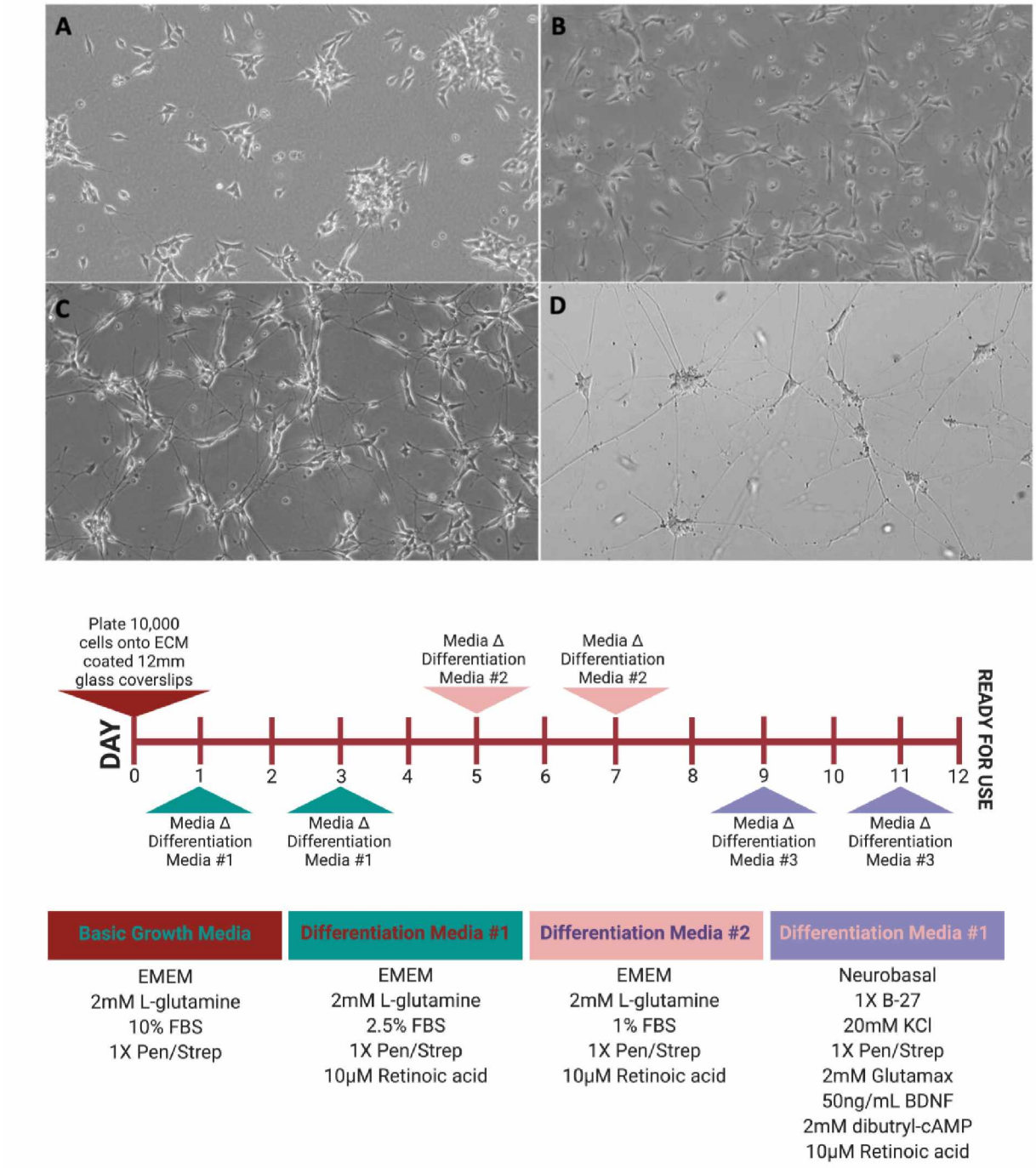

Figure 3.3. SH-SY5Y neuroblastoma cells exhibit varying morphology during the differentiation process. A. Undifferentiated SH-SY5Y cells are short and stubby, often growing in small clumps and lacking neuritic processes. Undifferentiated cells are maintained in medium containing 2.5% serum and 10 μM retinoic acid (RA) for four days. B. At the end of four days, cells have changed to exhibit more elongated cell bodies and longer processes. Cell are switched to medium containing 1% serum and 10

μM RA and maintained for an additional four days. C. At the end of four days, cells have developed more branched processes with more evidence of retracting cell bodies. At this point, cells are moved to medium composed of neurobasal, RA, and several neurotrophic factors while omitting all serum. D. After four days, cells exhibit long, exquisite neuronal branching with the small, rounded cell bodies characteristic of the neuronal phenotype.

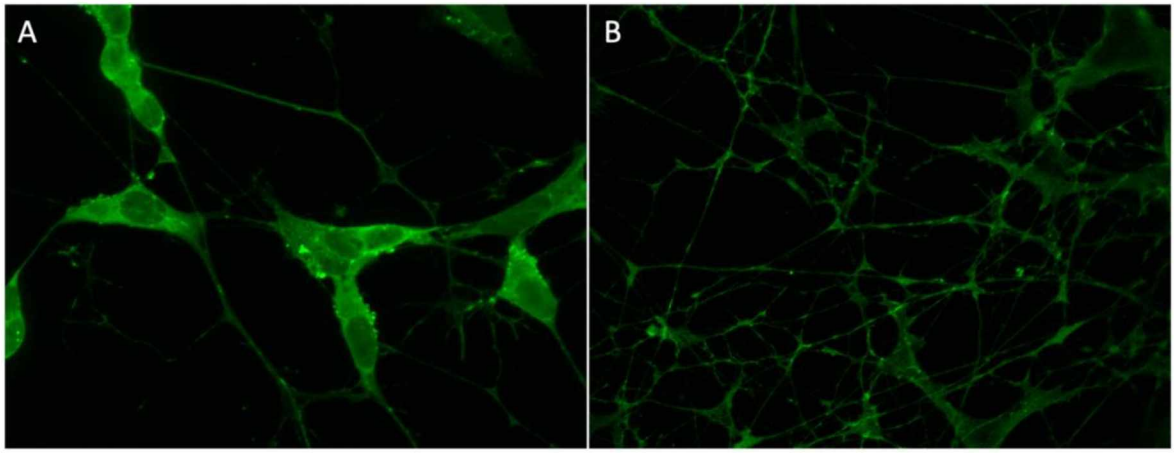

Figure 3.4. SH-SY5Y cells differentiated to Day 8 (D-8) and Day 12 (D-12) of the differentiation protocol do not form rods in response to gp120 exposure. Cells differentiated to D-8 (panel A) and D-12 (panel B) were exposed to 250pM dual-tropic gp120 for 18 hours before being fixed, immunostained against cofilin, and imaged at 600X magnification. D-12 cells exhibit a considerably denser network of neuritic processes as compared to D-8 cells.

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Chapter 4: Establishing a Protocol for the Direct Induction of Human Embryonic Stem-Cell Derived Glutamatergic Neurons: Towards A Model for Investigation of HAND

4.1. Abstract

Human embryonic stem cells (hESc) and inducible-pluripotent stem cells (iPSC) have emerged as potential solutions to the problems associated with the use of immortalized cell lines and animal models in the context of investigations of human disease pathologies. The capacity to generate primary neurons of human origin for use as cellular models would prove invaluable in better understanding the molecular mechanisms underlying numerous neurodegenerative diseases, from Alzheimer's disease to HIV-associated neurocognitive disorders. Here, we describe a method that allows for the direct induction of hESc to mature human neurons by the forced expression of the transcription factor neurogenin-2 (NgN2) with a lentiviral transduction and tetracycline-inducible expression of transduced genes driven by a tetO promoter. This method reliably produces human glutamatergic neurons with near 100% conversion efficiency and neuronal purity within 2 weeks of transduction. Neuronal cultures can be maintained long-term (40+ days) to allow maturation before use in downstream applications.

4.2. Introduction

As of the close of 2018, the number of people living with HIV/AIDS has reached 36.9 million globally, with an estimated 5,000 new infections occurring daily (UNAIDS, 2019). While progress towards the reduction of new infections has been frustratingly slow, advances in the treatment of disease have contributed to a decline in mortality rates and has raised the predicted life expectancy in people with HIV to near normal levels (Nakagawa et al., 2012). Despite this, the complications associated with long term infection, including the cognitive dysfunctions reported in upwards of 50% of infected individuals, continue to pose a public health challenge. These progressive neurological complications are referred to as HIV-1-associated neurocognitive disorders (HAND) and encompass a broad spectrum of cognitive, motor, and behavioral abnormalities. Despite the prevalence of HIV-associated cognitive dysfunctions, the molecular and cellular mechanisms underlying HAND are poorly understood but are likely to consist of a combination of indirect mechanisms involving the release of inflammatory mediators and viral proteins from infected glial cells and direct effects mediated by the interaction of neurotoxic viral proteins with neuronal receptors.

Research into the pathophysiological mechanisms underlying HAND and other neurodegenerative

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Authorship for this chapter is as follows: Lisa Smith and Thomas Kuhn.

diseases has been hindered by the lack of relevant human neuronal models. Many of the current *in vitro* approaches to understanding the pathogenesis of HAND are performed in transformed human immortalized cells lines or genetically manipulated murine primary neurons ("humanized mice"), owing to the expense and ethical issues involved in the use of primary neurons of human origin. However, use of immortalized cell lines come with many caveats including cells which exhibit neuro-potential but lack neuronal morphology, as has been observed in the human neuroblastoma SH-SY5Y cell line. While it has been demonstrated that SH-SY5Y cells can be differentiated to yield a homogenous population of neuron-like cells that can be used for investigation of CNS-relevant diseases, it remains that the tumor-origins of the cell line may result in genomic abnormalities that result in alternative functional responses to stimuli that must be considered when interpreting results obtained from these systems. Further, the use of primary mammalian neurons is limited by restrictions in propagation- once terminally differentiated into mature neuronal cultures, these cells can no longer be propagated. In vivo investigations of HAND are limited to animal models, frequently in transgenic rodent models whose non-human origins make it difficult to translate these results to true human pathogenesis.

Human embryonic stem cell (hESc) and human inducible pluripotent stem cell (hiPSC) technologies have emerged as promising approaches for the investigation of neurodegenerative disease in primary neurons of human origin. The application of varying differentiation protocols allows researchers to differentiate and expand hESc and hiPSCs into large quantities of functional neurons of specific subtypes pertinent to the pathologies under study. Numerous groups have reported success in establishing stem cell models for the investigation of human neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases as well as for the study of human neurotropic viruses, including Herpes Simplex and Varicella Zoster (Tousley and Kegel-Gleason, 2016; Xiao et al., 2016; Yang et al., 2016; Pourchet et al., 2017). Moreover, these model systems also carry potentially crucial epigenetic information, which is thought to play a vital role in disease pathologies. However, most methods for differentiating these cells are time-consuming with low conversion efficiency and lack reproducibility between different pluripotent cell lines used for the initial conversion of these cells into induced neurons (iN) (Hu et al., 2010).

Here, we describe a method for the direct induction of the H1- hESc line into functional iN cells by forced expression of a single transcription factor. A similar method has been previously demonstrated to convert both hESCs and hiPSCs into functional neurons with near 100% efficiency and purity. Neuronal induction of stem cells under this method rapidly and reproducibly generates functional iN cells in less than two weeks and provides a primary human neuronal model for the investigation of human neurodegenerative diseases, including HAND.

4.3. Protocol

4.3.1. General Considerations

1. H1-hESc have been derived from the inner cell mass of blastocytes and have been approved for use in federally funded research. These cells can be subjected to long term storage and amplified without affecting cell viability

2. These cells will grow very rapidly with a doubling time of ~12 hours. In contrast to most cell type culturing methods, H1-hESc cells will grow in small clumps forming islands in culture. These cells must be grown in colonies to maintain growth and pluripotency

3. Cells must be plated onto Matrigel-coated dishes; media components and substrates are critical to the induction of iN from these cells. See the **Table 1** for a list of reagents.

4.3.2. Matrigel Coating of 6-well plates and 12mm glass coverslips

1. Maintain Matrigel frozen as a 10x stock at -20°C; thaw on ice and keep cold until ready for use

2. Dilute Matrigel stock into DMEM/F12 at 1x concentration to desired volume. Add 1mL 1x Matrigel per well for 6-well plate, or 80uL per glass coverslip. Incubate for 1 hour at 37°C

3. Remove media and plate cells directly into wells or onto coverslips without washing

4.3.3. Plating H1-hESc maintenance cultures

1. Gently thaw one vial (0.5mL) hESc **only** until frozen media is in solution

2. Add 2mL of hESc growth medium (See **Table 2**) and gently mix

3. Spin cells for 3 minutes at 200xg_{max} and aspirate supernatant

4. Resuspend the cell pellet in hESc plating medium (see **Table 2**) (volume = 2mL/well plated)

5. Plate 2mL of the cell suspension per well

6. After 24 hours, hESc grow into small, clumped islands with cells exhibiting protrusions at the edges of islands

7. Transfer cultures to hESc growth medium with a complete media exchange. Clumps of cells will grow into islands with smooth edges/no or few protrusions

8. Allow cells to grow to ~80% confluence before re-plating

4.3.4. Passage of H1-hESc maintenance cultures

1. Wash cells twice with cold dissociation medium (see **Table 2**) using 1mL/well

2. Incubate cultures with 1mL dissociation medium at 37°C for 3-4 minutes. Islands will round up and visibly shrink in size

3. Remove dissociation medium and **gently** add growth medium (1mL/well)

4. Carefully aspirate medium and replace with 1mL growth medium/well

5. Dislodge cells by washing with a 1000μL pipette. Do **not** triturate cells

6. Spin cells for 3 minutes at 200xg_{max} and aspirate supernatant. Resuspend cell pellet in hESc plating medium for a total volume = (# wells X 6) X 2mL

7. Plate 2mL of cell suspension per well. After 24 hours, transfer cultures to hESc growth medium with complete medium exchange and note passage number

4.3.5. Freezing H1-hESc maintenance cultures

1. Follow steps 1-6 described in "Passage of H1-hESc maintenance cultures"
2. Resuspend cell pellet in BAMBANKER (see **Table 1**) using 1mL/well.
3. Aliquot 500uL cell suspension into cryotubes
4. Place at -80°C overnight before transferring to liquid nitrogen for long term storage

4.3.6. Preparing H1-hESc cultures for differentiation

1. Follow steps 1-3 described in "Plating H1-hESc maintenance cultures"
2. Resuspend cell pellet in 4mL hESc plating medium
3. Plate 2mL of cell suspension per well (in a 6-well plate). After 24 hours, cells will grow in small, clumpy islands with protrusions at edges of islands
4. Transfer cultures in complete media exchange to hESc growth medium
5. Re-plate hESc cultures when reaching 80% confluence at a ratio of 1:3 (1 well → 3 wells of 6-well plate) in plating medium
6. After 24 hours, transfer hESc cultures to growth medium in a complete medium exchange. Grow cultures to ~80% confluence
7. Dislodge hESc and triturate harshly into single cell suspension. Re-plate hESc 1:1 with plating medium into a Matrigel coated 6-well plate
8. After 24 hours, perform a complete medium exchange to growth medium and let cultures grow until confluent (**Day -2**)

4.3.7. Lentiviral transformation of hESc

1. Prepare lentiviral particle in growth medium at desired viral dilution
2. Add lentiviral particle solution to cultures with a complete medium exchange (**Day -1**)
3. After 24 hours, perform a complete medium exchange to N3 medium (See **Table 2**) containing doxycycline (**Day 0**). At this time, it is also possible to maintain cells in mTeSR complete with doxycycline (**See Table 2**).
4. After 24 hours, transfer cultures to N3 medium containing doxycycline and puromycin (See **Table 2**) by complete medium exchange. (**Day +1**).
5. Perform a complete medium exchange daily using N3 medium containing doxycycline and

puromycin (**Day +2** through **+5**). Cells display vigorous process formation together with considerable cell death of non-infected cells driven by puromycin selection.

6. At **Days +5** to +7, prepare induced neuron (iN) cells for re-plating

4.3.8. Long-term maintenance of iN cultures

1. Aspirate medium and wash cultures with PBS/1mM EDTA twice

2. Incubate with Accutase (See **Table 1**) for 3-5 minutes at 37°C, 1mL/well

3. Gently dislodge iN cells from wells and transfer to 15mL conical tube. Add 1mL N3 medium for each well of iN cells

4. Spin cells for 3 minutes at 200xg_{max} and remove supernatant

5. Resuspend iN cells in N3 medium containing 5% FBS and 1x doxycycline (See Table 1) as follows:

- Combine cells collected from one 6-well plate with 1.5mL of N3 medium containing serum and doxycycline.
- Add 1mL mouse P3 glia (for a total volume of 2.5mL)

6. Plate iN/glia cell suspension- 100□L per glass coverslip. Allow cells to adhere for 1-2 hours

7. Supplement each well with 500□L N3 medium with serum/doxycycline

8. After 24 hours, perform complete medium exchange using N3 medium with 2.5% heat inactivated FBS and 1x doxycycline

9. Perform a half medium exchange every 3 days. After day 3 to 4, include FdUr or AraC in the medium to inhibit proliferation of non-neuronal (non-infected) cells

10. At Day 7 after re-plating, perform half-medium exchange with Neurobasal media, omitting doxycycline, for a gradual transition to serum free conditions

4.4. Representative results

Many existing protocols for the differentiation of hESc into human iN are lengthy, cumbersome procedures that make large-scale studies difficult and have been complicated by relatively low yields and low efficiency. The protocol described above allows for the direct conversion of hEScs into functional human induced neurons in less than 2 weeks. Upon initial plating in media containing ROCK inhibitor Y-27632 (Table 2), hES cells appear as clumpy islands with multiple spiky protrusions along the perimeter (Figure 2 A, B). ROCK inhibition Rho kinase has been demonstrated to support maintenance of stem cell phenotype prior to differentiation and improve the survival of hESc monolayers at the initiation of differentiation protocols (Vernardis et al., 2017). Transferring hESc cultures to growth medium (Day -2) removes ROCK inhibition and cell edges begin smoothing as cells adhere to Matrigel coating and continue to proliferate (Figure 2 C, D). When cultures have reached ~80% confluence, cells are infected

with lentiviral particles expressing the neuronal transcription factor neurogenin-2/puromycin resistance gene fusion protein, EGFP, and rtTA (reverse tetracycline-controlled transactivator) at the desired multiplicity of infection (MOI) (Day -1). 24 hours post-infection, cultures are exposed to doxycycline, a tetracycvline derivative capable of activating transcription at the TetO promoter (Day 0) (Figure 3). Cultures are maintained in the presence of doxycycline for the remainder of the induction protocol. On Day +1, cultures are exposed to both doxycycline and puromycin. The NgN2/puromycin resistance gene fusion protein ensures only successfully transduced cells expressing the NgN2 transcription factor will survive puromycin selection, allowing for the generation of a pure population of induced neurons. Following activation of doxycycline-induced gene expression at Day 0, transduced cells display rapid morphological changes and display a characteristically neuronal phenotype within 2-4 days of transcriptional activation. By Day +5 and +6, cells exhibit round cell bodies and continually extending neurite-like processes characteristic of human neurons (Figure 4). At this point, iN cells can be used for early down-stream applications, or more typically— are re-plated onto a feeder layer of mouse glia cells, to support healthy long-term cultures of hiN. Glia proliferation is controlled during long-term culture with the inclusion of 5-fluoro,2'-deoxyuridine (FdUr) or cytosine β-d-arabinofuranoside (AraC), potent inhibitors of mitotic cell division. It has been previously demonstrated that while iN induced via the forced expression of NgN2 begin exhibiting typical excitatory signals after 28 days in culture, neurons exhibit changes in protein expression as maturation continues. Therefore, continued culture to Day +40 through +60 is recommended to generate mature neurons for experimentation. Indeed, a recently conducted proof-of-principal experiment failed to trigger the formation of actin-cofilin rods in induced neurons when stimulated shortly after re-plating with known rod inducers (data not shown). As rods have been identified in hippocampal and cortical neurites of post-mortem brains of Alzheimer's patients, this is hypothesized to be a consequence of an 'immature' neuron that may not yet be expressing all components for signaling rather than an inability of human neurons to form rods (Minamide et al., 2000). To re-assess rod formation in induced neurons, cultures are currently undergoing long-term maintenance for experimentation after 40+ days of maturation in culture.

4.5. Discussion

Though neuronal cell lines and animal models have been widely used for the in vitro investigation of neurodegenerative disease, any results obtained from these models requires consideration of the differences between neuronal cell lines and primary neurons and that using neurons from non-human origins may affect experimental outcomes and limit the applicability of results, particularly for a human host-specific virus such as HIV. Numerous studies have identified differences between neuronal cell lines— even cell lines differentiated into more neuron-like cells— and primary neurons, including

changes in receptor expression and function, as well as differential responses following exposure of neurotoxins (LePage et al., 2005; Edwards et al., 2007; Shipley et al., 2017). Stem cells have emerged as promising tool in the field of neurodegenerative research, allowing researchers to model brain disease directly in human neurons in instances where the availability of brain tissue is restricted. Here, we have demonstrated the rapid neural induction of hES cells with cultures exhibiting neuronal morphology by 6-days post induction. This direct induction of neuronal phenotype requires the forced expression of neuronal transcription factor Ngn2, introduced to hESc cultures through the use of lentiviral particles expressing NgN2, puromycin resistance, EGFP, and rtTA genes under the regulation of the Tet-On system for doxycycline induced gene expression (Zhang et al., 2013; Das et al., 2016) and yields neuronal cultures of near 100% purity. Importantly, this method of direct induction has been demonstrated to convert iPSC to iN with similar success (Chanda et al., 2014) and can potentially be used to generate primary neurons with regional CNS specificity by modifying transcriptional activators transduced.

The approach outlined here is directed toward the establishment of a relevant neuronal model of human origin for the investigation of HIV-neuron interaction with a particular aim of evaluating the role of HIV envelope glycoprotein gp120 in the induction of neuronal cofilin-actin rods in primary human neurons. Gp120 serves as a viral surface protein recognizing host CD4 and chemokine co-receptors CCR5 and CXCR4 and is among the most potent HIV neurotoxins with a well-documented role in neurodegeneration mechanisms underlying HIV-associated neurocognitive disorders. We have previously described a pathway to the formation of rods activated by gp120 interaction with HIV co-receptors CCR5 and CXCR4 in rodent hippocampal and cortical neurons. Generation of rod-like inclusions is a cellular response to oxidative stress resulting from the rapid dephosphorylation (activation) of cofilin and subsequent saturation of actin filaments, inducing bundling into rod-like structure and have been implicated in the synaptic dysfunction underlying other neurodegenerative diseases (Bamburg et al., 2010; Goyal et al., 2013). However, results obtained from animal models of neurodegeneration do not necessarily recapitulate human disease, and indeed, attempts at translating results from animal models to human systems often end in failure (Zeiss, 2017; Dawson et al., 2018; Ransohoff, 2018). A human primary neuronal model for the investigation of gp120-mediated neurotoxicity is the first step in overcoming current barriers to research by establishing a species-relevant model while avoiding the limitations associated with the use of immortalized cell lines. The method described here for the induction of human primary neurons has the potential to improve our current understanding of the molecular mechanisms of HAND and better characterize a novel molecular pathway leading to synaptic dysfunction previously undescribed for HIV.

4.6. Figures and Tables

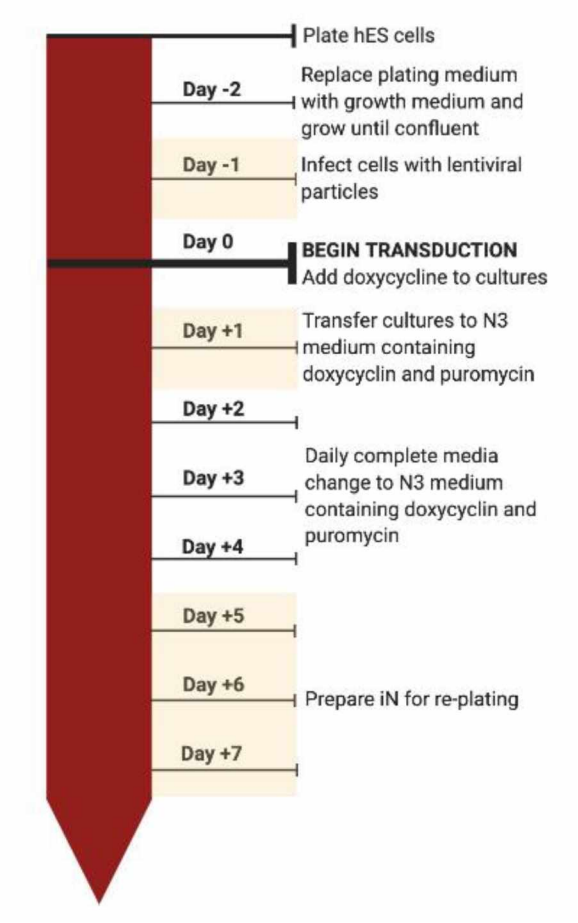

Figure 4.1. Timeline to induction of human induced neurons (hiN). The induction of human embryonic stem cells (hESc) to hiN cells occurs rapidly with iN cells visible in culture by Day +6. hESc cells are grown to confluence and infected with lentiviral particles expressing NgN2-EGFP, puramycin resistance, and rrTA/TetO for one day before switching to medium containing Tet activator doxycycline (Day 0). On Day 1, cells are transferred to medium containing both doxycyclin and puromycin to select for transduced cells. Cells are maintained in this medium with daily full media changes until Day 5+, at which point hiN can be prepared for re-plating.

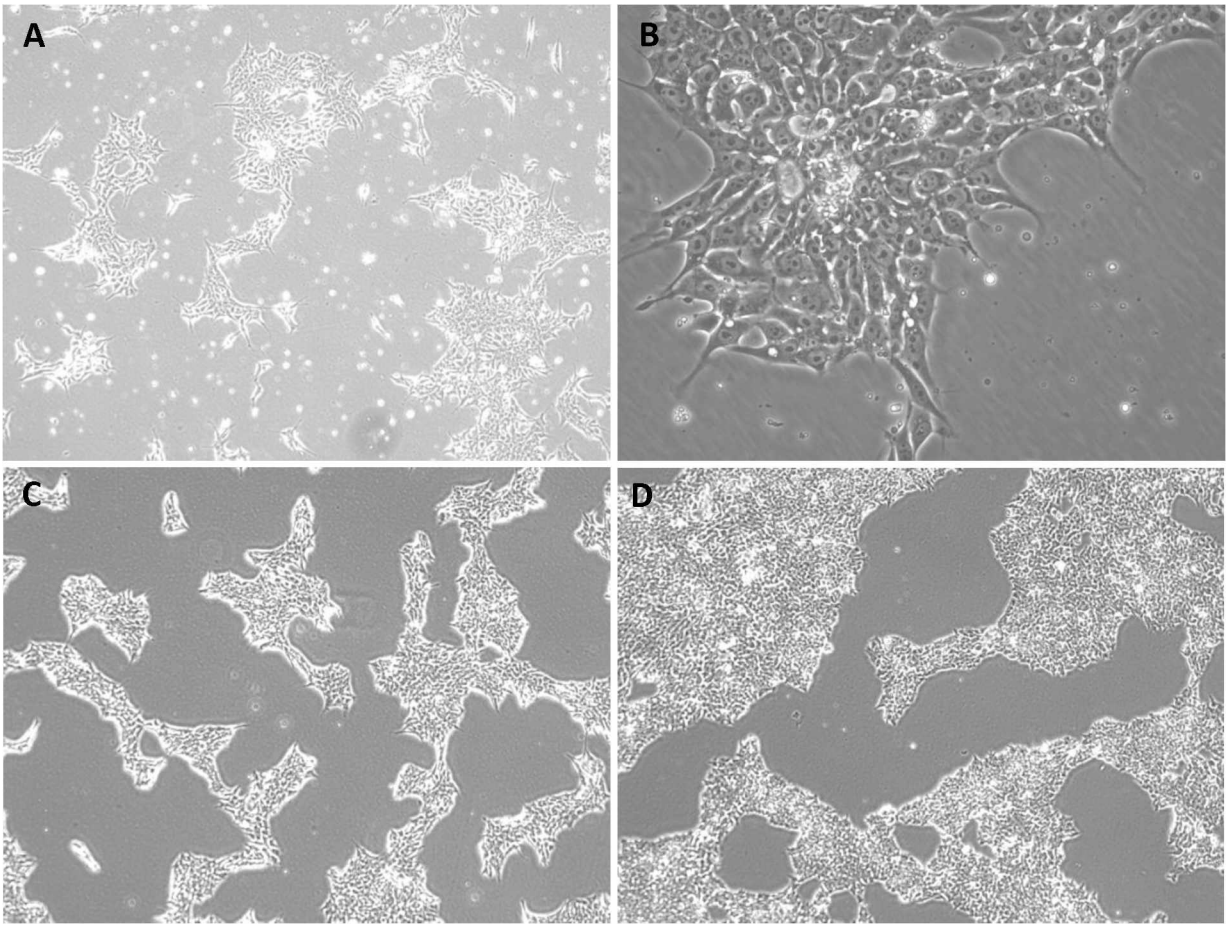

Figure 4.2. Stem cell morphology when initially plated in hESc plating medium containing the ROCK inhibitor Y-2763 (A, B) and in hESc growth medium without the inhibitor (C, D). After initial plating, cells form small clumps, growing into islands of increasing diameter as they adhere to Matrigel coating. In the presence of Y-2763, cells along the perimeter of the islands show multiple small protrusions resembling spikes (A, B). Figure 2B shows a 40X enlargement of protrusions. Removal of Y-2763 from growth medium leads to cells becoming more rounded with smooth edges maintained as the islands grow more confluent (C, D).

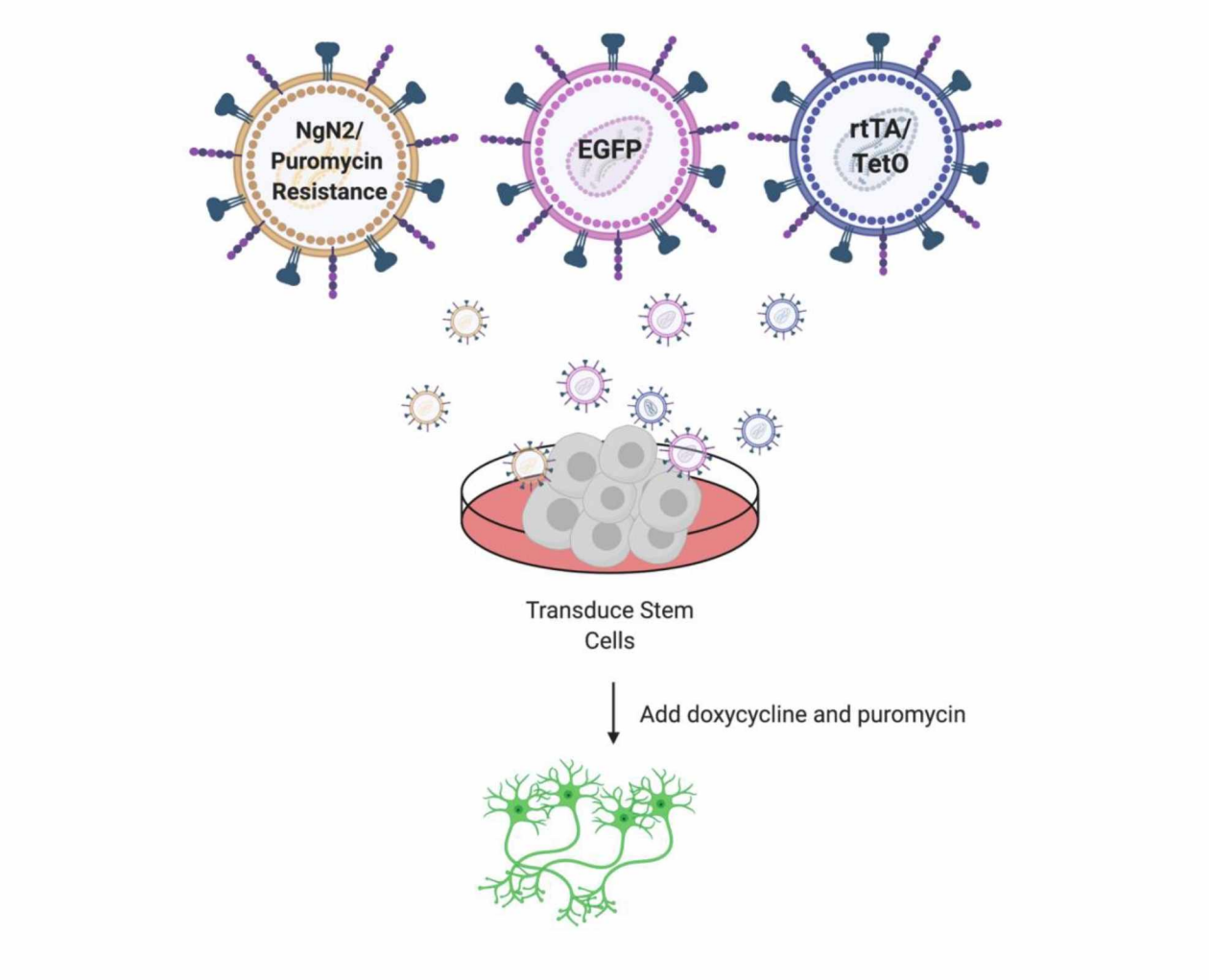

Figure 4.3. Schematic of direct induction of hESc to hiN using lentiviral vectors. hESc cells were transduced with 3 viruses with one expression neuronal transcription factor fusion protein NgN2/puromycin resistance, another expressing EGFP, and the final expressing the reverse tetracycline-controlled transactivator (rrTA) (Day -1). One day after infection, cells are exposed to doxycycline to initiate expression of transduced genes via Tet-On driven transcriptional activation (Day 0). Beginning Day +1, cells are exposed to medium containing both doxycycline and puromycin to select against non-transduced hESc. By Day +6, hiN show neuronal process outgrowth and are ready to be re-plated for downstream applications.

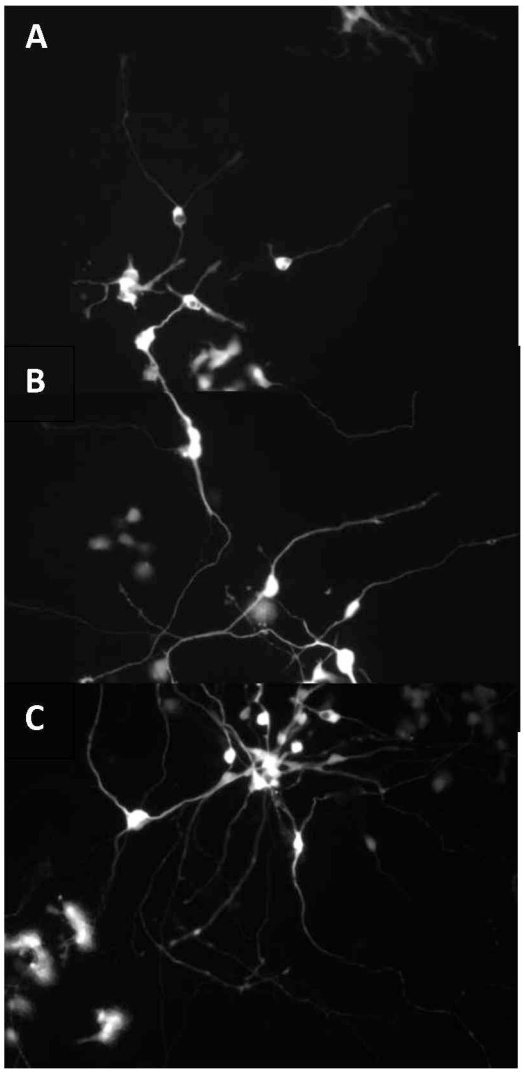

Figure 4.4. Final steps in the direct induction of hESc to iN cells— morphology of transduced cells on Day +4, +5, and +6 (A-C) of the protocol. After induction, the cells rapidly differentiate to demonstrate morphology characteristic of a neuronal phenotype. Note that by Day +5 and +6, cells have already begun to exhibit neuronal process outgrowth (B, C).

Table 4.1. Media components and vendor information.

Component	Vendor	Catalog Number	Storage
Matrigel™	Corning	356234	-20℃
mTeSR Plus (400mL basal/100mL supplement)	Stemcell Technologies	5825	4℃
DMEM/F`12	Fisher	11320082	4℃
Hyclone FBS heat-inactivated	Fisher	3007003H1	-20℃
Y-27632 (ROCK Inhibitor)	Stemcell Technologies	72302	-20℃
0.5 M EDTA	Lonza AccuGene	51234	4℃
Doxycycline (1000X)	Sigma Aldirch	D1822	-20℃
Puromycin (1000X)	Thermo Fisher	A1113802	-20℃
N2 Supplement (100X)	RND Systems	AR009	-20℃
B27 Supplement (50X)	Gibco	17504-044	-20℃
Insulin	Sigma Aldrich	13536	-20℃
Arabinoside C	Sigma Aldrich	1162002	-20℃
5'-fluoro-deoxy-uridine	Sigma Aldrich	F8791	-20℃
BAMBANKER	Fisher	NC9582225	2-10℃
Accumax	Innovative Cell Technologies Inc.	AM-105	-4℃

Table 4.2. Media recipes

Component	Instructions
hESc Growth Medium	Thaw mTeSR plus supplement overnight at 4℃. Combine with 400mL basal mTeSR Plus and store at 4℃. Add Pen/Strep at 100 U/mL or 5mL/500mL medium. Warm to 37℃ before use.
hESc Plating Medium	Dilute Y27632 (ROCK inhibitor) into hESc growth medium at 5µM immediately before plating. Do not store.
Dissociation Medium	Dilute 0.5M EDTA in PBS to 1mM
N3 Medium for iN (50mL)	48mL DMEM/F12 0.5mL N2 supplement (100X) 1mL B27 supplement (50X) 0.5mL penicillin/streptomycin (100X) 1mg insulin 50µL doxycyline (1000X) ---when called for— 50µL puromycin (1000X)
Neurobasal (50mL)	49mL Neurobasal 1mL N21max supplement (50X) 0.125mL Pen/Strep (10,000 U/mL) 0.125 mL Glutamax

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Chapter 5: General Conclusions

5.1. General Overview

Since its emergence in the early 1980s, the human immunodeficiency virus (HIV) and resulting acquired immunodeficiency syndrome (AIDS) have been responsible for 35 million deaths and more than 70 million infections worldwide (UNAIDS, 2019). The implementation of combination antiretroviral therapy (cART) as the primary treatment regimen has shifted HIV/AIDS from an acute infection to a chronic disease with new challenges emerging in the form of associated comorbidities that can have profound impacts on quality of life. Included among these are the cognitive, motor, and behavioral abnormalities experienced by upwards of 50% of infected individuals, referred to as HIV-1-associated neurocognitive disorders (HAND)(Kaul et al., 2005; Saylor et al., 2016).

HAND develops over the course of infection in people living with HIV (PLWH) and is classified into three categories according to the degree of cognitive impairment— asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MNI), and the most severe, HIV-associated dementia (HAD). Prior to the introduction of antiretroviral therapy in the early 1990s, 20-30% of those infected with HIV developed HAD (Ciccarelli, 2020). Characterized by subcortical dementia with impairments in both cognitive and motor functions, brain atrophy, and neuronal loss, the incidence of HAD has seen a dramatic decline (~2% of the population of PLWH) over the last two decades (Saylor et al., 2016). While HAD has declined, the milder forms of HAND persist and are expected to increase in prevalence with the aging population of PLWH. Those affected by ANI and MNI often present with symptoms similar to cognitive impairments arising from other causes and include shortened attention span, mood disorders, confusion, and impairments in fine motor skills (Ciccarelli, 2020). These impairments, while mild in comparison to HIV dementia, continue to have profound impacts on the quality of life for those affected. Not surprisingly, neuropathology underlying these milder forms of HAND differs from HAD, and strikingly, post-mortem examinations no longer observe the encephalitis and widespread neuronal loss previously reported for HAD (Gelman, 2015; Saylor et al., 2016). Rather, HAND in the post-cART era is likely to be the result of functional alterations in neurons arising from dysregulation of neuronal homeostasis and impaired neuronal plasticity characterized by synaptodendritic damage (Smith et al., 2018).

HIV invades the central nervous system (CNS) within weeks of initial viral exposure and remains a site of persistent infection regardless of adherence to antiretroviral regiment (Edén et al., 2007; Sturdevant et al., 2015). HIV infection of the CNS is associated with activation of microglia and astrocytes, as well as the induction of inflammatory and neurotoxic insults, which are suspected to stimulate a progressive

synaptic degeneration manifested in cognitive decline. Despite the increasing prevalence of HAND, the underlying molecular and cellular mechanisms are poorly understood and are thought to consist of a combination of direct viral infection of cells of the CNS and indirect mechanisms involving inflammatory cytokines, metabolites, and neurotoxic effects of viral proteins, including the envelope glycoprotein gp120. The neurotoxicity of gp120 has been widely reported, with a demonstrated role in inducing neuronal apoptosis, dysregulation of neuronal receptors, and notably— synaptic and dendritic damage (Lipton et al., 1991; Kaul et al., 2001; Chen et al., 2002; Iskander et al., 2004; Melli et al., 2006).

Intriguingly, HAND and Alzheimer's disease share many common neurotoxic pathways and individuals with HAND show increased synthesis of amyloid-β (Aβ) as well as increased phosphorylation of Tau (Canet et al., 2018). Both Aβ and Tau are neurotoxic proteins implicated in the progression of Alzheimer's disease with a role in mediating synaptic dysfunction. Notably, we have previously identified a neurotoxic signaling pathway stimulated by Aβ oligomers and proinflammatory cytokines that contributes to the synaptic dysfunction observed in Alzheimer's disease. This pathway requires the expression of cellular prion protein (PrPc) and the activity of NADPH oxidase-2 (NOX2) embedded in coalesced lipid raft microdomains. Subsequent ROS generation is causative to the formation and accumulation of rod-like cofilin-actin inclusions (rods) in neuronal processes, which contribute to synaptic dysfunction via sequestration of cofilin and actin from dendritic spines, as well as disruption of vesicular transport resulting from occlusion of neurites containing rods (3, 4). Lipid rafts, transient cholesterol and sphingolipid enriched microdomains in the plasma membrane, serve as coalescing signaling platforms promoting clustering of receptors and components of receptor-activated signaling cascades, thereby initiating cellular signaling (5). Protein association with lipid rafts as a mechanism for regulating signaling has been observed for a number of neurodegenerative diseases, including the pathway to rod formation described for Alzheimer's disease (6). Notably, HIV not only infects host cells via binding of gp120 to the CD4 receptor and CCR5/CXCR4 co-receptors localized in host-cell lipid raft domains but gp120 exposure of neurons similarly induces enhanced lipid raft coalescence and activation of NADPH-oxidase mediated generation of ROS.

Given the numerous similarities between HAND and Alzheimer's disease, we hypothesized that gp120 interaction with lipid-raft localized co-receptors CCR5 and CXCR4 would similarly induce the formation of actin-cofilin rods through the same pathway as Ab. To test this hypothesis, we generated 3 questions (aims) we wished to address:

1) What are the consequences of distinct gp120 co-receptor tropism on the generation of rod-like cofilin-actin inclusions in primary rodent neurons?

2) Does gp120 induces ROS and cofilin-actin rods in SH-SY5Y human neuroblastoma cells?

3) Can mature human neurons derived from human embryonic stem cells serve as a more relevant model for the investigation of gp120 induced actin-cofilin rods?

Chapters 2, 3, and 4 of this dissertation address these aims, and the results from each chapter are briefly discussed below:

5.2. Chapter 2: Direct Interaction of HIV gp120 with Neuronal CXCR4 and CCR5 Receptors Induces Cofilin-Actin Rod Pathology via a Cellular Prion Protein- and NOX- Dependent Mechanism

The experiments in Chapter 2 explored the ability of gp120 to induce the formation of cofilin-actin rods via a PrP^C / NOX2 dependent pathway, and further characterized the induction of rods in the context of gp120 tropism. Our results reveal for the first time gp120-mediated induction of cofilin-actin rods as a novel mechanism underlying synaptic dysfunction in HAND. Exposing E16 mouse hippocampal neurons to all strains of gp120 tested (dual-, R5-, and X4-tropic) led to a significant increase in number of rods present in neurites. We also provide evidence that gp120-mediated rod induction is dependent on the activation and subsequent generation of superoxide by NOX2, as well as the expression of PrP^C in lipid rafts. Though we demonstrate the involvement of both CCR5 and CXCR4 co-receptors in the activation of this pathway, our results suggest that there are receptor-specific differences in the neuronal response to gp120 induced signaling. Both R5- and X4- tropic strains of gp120 induced a significant rod response; however, the inhibition of CXCR4 mediated signaling alone rescued neurons from rod induction. In R5-tropic strains, inhibiting the CCR5 receptor resulted in a nominal decrease in the number of rods detected, suggesting that blocking gp120/CCR5 signaling achieves only a partial block of rod induction. Recent experiments have shown Ab-mediated rod induction is inhibited by maraviroc, the CCR5 receptor antagonist (data not shown). Importantly, the concentration of inhibitor used by investigators in this study was 100X the concentration we used to inhibit gp120 interaction with the receptor. Our partial block may be due to insufficient inhibitor concentration and warrants further study. These results identify a previously undescribed pathway to synaptic dysfunction mediated by gp120 highlighting new targets for therapeutic intervention.

5.3. Chapter 3: Lipid Raft Coalescence Links Gp120 Induced Oxidative Stress to Neurodegeneration

Having demonstrated the gp120-mediated induction of rods in E16 murine mouse hippocampal neurons, we next sought to observe the same pathology in neurons of human origin. We first exposed SH-SY5Y human neuroblastoma cells to gp120 and quantified the response by the increase in lipid raft size and stability (coalescence). We next quantified ROS production and confirmed NOX2 activity by measuring the response of neurons to gp120 exposure over time and quantifying the amount of NOX2 cytosolic subunit $p67^{PHOX}$ associated with the plasma membrane. We found SH-SY5Y responded to

gp120 exposure with a dose-dependent increase in lipid raft coalescence and a corresponding activation of NOX2. This activation of NOX2 induces a rapid rise in ROS, with superoxide levels elevated over control up to 2.5 hours post-exposure. Given the similar response to gp120 between the neurons of rodent and human origin, we then used differentiated SH-SY5Y cells to evaluate rod induction by gp120 in a human model. We were unable to detect any rod formation, as discussed in Chapter 3. However, it is possible that the reportedly low levels of endogenous PrP^C expressed by these cells is a contributing factor, as the expression of PrP^C is required for the induction of rod formation. These results suggest that SH-SY5Y cells respond to gp120 in a manner similar to what is seen for the mouse neuronal respond to gp120. If the absence of detectable rods in SH-SY5Y is truly due to low or absent expression of PrP^C, transfection or other genetic manipulation to force the expression of the protein may be a means of generating a functional human neuronal model for investigation rod pathology in HAND and other neurodegenerative disease.

5.4. Chapter 4: Establishing a Protocol for the Direct Induction of Human Embryonic Stem-Cell Derived Glutamatergic Neurons: A Model for Investigation of Hand

While SH-SY5Y cells provide an attractive alternative to murine cells for the investigation of the rod induction pathway, it remains that the immortal-cell line background can call into question the relevancy of results. Advances in stem cell technology have offered a potential solution to the problems associated with the use of non-human models and cell lines to investigate human disease. Many protocols for stem cell differentiation are time-consuming, complicated and affected by low yield and lack of reproducibility. In this chapter, we describe a method for the direct induction of human embryonic stem cells to human neurons. Using this method, the forced expression of transcription factor neurogenin-2 under control of TetOn inducible expression plasmid system generated human glutamatergic neurons with near 100% conversion in less than 2 weeks. These human neurons provide a new tool for investigating HAND in a primary human neuronal model.

5.5. Concluding Remarks

The results presented here highlight a novel pathway to synaptic dysfunction mediated by HIV glycoprotein gp120 directly interacting with co-receptors CCR5 and CXCR4 on the neuronal membrane of murine neurons. We further demonstrate that gp120 is capable of initiating lipid-raft coalescence and generation of superoxide by NOX2- critical steps in the pathway to rod induction observed for not only gp120 mediated rod formation in rodent neurons, but also rod induction by Alzheimer's associated Aβ peptide oligomers, in the human neuroblastoma SH-SY5Y cell line . Though most current *in vitro* models for investigating rod formation rely on the use of murine primary neurons, it remains that rodents are not

humans, and accordingly, to be relevant to human disease rod induction must be recapitulated in human neurons. While it is known that human neurons are capable of producing pathologic rods, as evidenced by post-mortem examinations of Alzheimer's brains where the presence of rods were detected only in diseased brains, to our knowledge rod induction has not been demonstrated in an *in vitro* human model (Minamide et al., 2000). Because SH-SY5Y cell scan be differentiated to a more neuronal phenotype exhibiting long, neuritic processes, we sought to confirm gp120 is capable of inducing rods in a human model. While we were unable to detect rod induction in the experiments described here, it is still possible that this cell line could be developed as a neuronal model for rod investigation in an easily accessible cell line. In addition to the development of an SH-SY5Y model for rod investigation, we also sought to establish a primary human neuronal model through the differentiation of human embryonic stem cells. The use of stem cells has emerged as a powerful tool in the field of neurodegeneration, allowing researchers to not only model brain disease directly in human neurons in instances where the availability of brain tissue is restricted. We demonstrate the successful generation of human glutamatergic neurons employing a method of direct induction yielding neuronal cells within 2 weeks. We are currently awaiting maturation of these neurons before evaluating for the expression of gp120 co-receptors and the ability to induce rods in response to known rod inducing stimuli. The PrP^C/NOX2 dependent pathway to rod induction described here highlights several potential new targets for therapeutic approaches to the management of not only HIV associated neurocognitive disorders, but other neurodegenerative diseases in which rod induction may underly synaptic dysfunctions.

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